

HAL DOWNEY

ANNALS OF THE NEW YORK ACADEMY OF SCIENCES

VOLUME 59, ART. 5 PAGES 665–1070

March 24, 1955

Editor

ROY WALDO MINER

LEUKOCYTIC FUNCTIONS*

Conference Co-Chairmen

ALBERT S. GORDON, JOHN W. REBUCK, AND ROBERT S. SPEIRS

Consulting Editor

ALBERT S. GORDON

CONTENTS

*This series of papers is the result of a Conference on *Leukocytic Functions* [illegible] held by the Section of Biology of The New York Academy of Sciences May 27, 28, and 29, 1954.

Part V Chemical and Metabolic Aspects of Leukocytic Activity

DEDICATION TO DOCTOR HAL DOWNEY

By Oliver P Jones

Department of Anatomy School of Medicine University of Buffalo Buffalo N Y

We were proud to honor Doctor Hal Downey by dedicating to him the Conference on Leukocytic Functions on which this monograph is based in recognition of his influence on hematology as a teacher as an editor and as an investigator In doing so we realize that 50 years ago he dedicated himself to the study of the biology of blood cells and to the field of hematology with such an insatiable curiosity that it has continued into the years following his academic retirement It was a fortuitous circumstance which kindled the spark of interest in hematology in him After completing his master s degree in 1904 Doctor Downey was assigned the problem of investigating the genitourinary system of the Spoon bill Sturgeon (*Polyodon spathula*)—a ganoid fish having many primitive characteristics found in the Mississippi River It was soon discovered that the kidney of *Polyodon* was the chief hematopoietic organ Doctor Downey became so interested in this phase of the problem that he decided to investigate the hematopoietic tissues rather than the genitourinary system As a result he was launched into the field of hematology and published his first article on The Lymphatic Tissue of the Kidney of the *Polyodon spathula* [1]

Doctor Downey s enthusiasm for hematology was so great that he read every article available at the time which had any bearing on that subject As a result he visited Europe in order to study with two excellent hematologists—the one a clinician and the other an anatomist At the Anatomical Institute in Strassburg he worked with Doctor Franz Weidenreich on a problem which was destined to become a cornerstone for future investigations In their article Über die Bildung der Lymphocyten in Lymphdrusen and Milz [2] Downey and Weidenreich showed for the first time that there is a genetic relationship between the reticulum and the lymphocytes This work which was done on laboratory animals was later confirmed by Maximow in human material Although Downey studied the reticulum in his work on *Polyodon* one cannot help but feel that his work with Weidenreich was responsible for his interest in the derivation of leukocytoid lymphocytes in lymph nodes from patients with infectious mononucleosis and for his recognition and understanding of the various types of leukemic reticuloendotheliosis Today some hematologists have either overlooked or have forgotten that Doctor Downey published a classical piece of research based entirely on sectioned material

Perhaps a very definite contributing factor in Doctor Downey s bias for the *dry smear method of hematological study was the fact that he spent some time* studying in Pappenheim s laboratory One has only to glance at some of Pappenheim s lithographic plates to understand the tremendous emphasis which he placed upon a detailed study of nuclear pattern Doctor Downey thus received the best possible training from two famous hematologists who had divergent views as to the manner in which blood and blood forming organs should be studied

It was during Doctor Downey s stay in Pappenheim s laboratory that Doctor J H Wright made a tour of Europe demonstrating the technique for showing the megakaryocytic origin of blood platelets Bunting of Wisconsin had confirmed Wright s work in 1909 and 1911 so that it was generally accepted in this country After returning from Europe, Doctor Downey also confirmed Wright's work and published a paper, chiefly for the benefit of foreign hema tologists few of whom accepted the results of Wright s work and to check Dominici s theory that platelets were formed as cytoplasmic buds from lym phocytes[3] This work obviously paved the way for subsequent investigation by Doctor Downey and his students of the origin of megakaryocytes in the spleen and liver in a case of atypical myelosis[4] and for the study of a case of myeloid megakaryocytic hepato splenomegaly[5]

Doctor Downey was also interested in the other phase of the platelet prob lem namely the relation of cytoplasmic buds from lymphocytes to platelets These buds were found to be quite numerous in lymph vessels but not very numerous in the thoracic duct Doctors Downey and Weidenreich had ob served that these buds were given off by lymphocytes of the germ centers follicles and interfollicular tissue and sinuses The relation to the platelet problem came about because these buds appeared to be identical in structure with blood platelets Doctor Downey was able to show by means of a special technique that these bodies do not contain granules characteristic for blood platelets

In this connection it is interesting to know that Doctor Downey thought the buds from lymphocytes might have something to do with the coagulation of lymph He wrote to Professor Howell concerning this matter but never heard whether Howell ever gave it any consideration Although Howell may not have thought so much of these hyalin bodies other investigators have seen in them a morphological basis for the explanation of antibody formation Ehrich and Harris[6] support their lymphocytic theory of antibody formation by utiliz ing the observations made by Doctors Downey and Weidenreich Dougherty Chase and White[7] correlate the production of gamma globulin with the budding of lymphocyte cytoplasm

Doctor Downey was first thrust in the midst of a controversial medical prob lem by collaborating with Mandlebaum in a study of the histopathology and biology of Gaucher s disease[8] In subsequent years he published several arti cles dealing with hematological problems allied to the fields of internal medicine and pathology Three of these articles were fundamental and have been re ferred to as classic examples of morphologic hematology Doctor Downey s work with McKinlay on the alterations of lymphocytes in cases of infectious mononucleosis is even more important today than it was 31 years ago for we now know that this disease causes changes in the lymphocytes even when the heterophil agglutination test may be normal The articles on the myeloblast are also of a most fundamental nature It is only through a complete under standing of this problem that one is able to interpret the differences in the re generation of lymphocytes under normal conditions and in acute leukemias The third important and fundamental article is his chapter on monocytic leu

kemia and leukemic reticuloendotheliosis published in the *Handbook of Hematology* edited by Doctor Downey

Doctor Downey also published basic papers on the development of the spleen and lymph nodes and two articles on the reaction of blood and tissue cells to acid colloidal dyes Although the latter is a subject which has received less recognition than some of the other published articles Doctor Downey has had a number of graduate students study connective tissue from time to time In one particular study he and his students were able to show that the supposedly highly differentiated fibroblast could transform into histiocytic elements and macrophages This potentiality was particularly true after injections of typhoid vaccine These *in vivo* experiments preceded by quite a number of years the *in vitro* experiments which showed a similar response of fibroblasts

Doctor Downey was definitely a graduate school teacher He enjoyed and lived for research and showed little interest in any student lacking a flair for research Doctor Downey s technique for training graduate students may be summarized in a quotation by Bashford Dean An elaborate bibliography is the strongest scaffolding upon which any research can be built He was acquainted with all hematological literature of any importance and knew thoroughly the various theories and he expected as much of his students Doctor Downey was also a stickler for good technique He would lose all interest in a particular case or problem if the slides were poorly stained His knowledge of what good technique means was undoubtedly responsible for his being selected to contribute to McClung s text on microscopic technique

It is impossible to estimate how many technicians graduate students internes and residents have come under Doctor Downey s influence through his course in hematology which was unique and unparalleled in this country if not in the world Before closing it is only fitting to relate another fortuitous circumstance which perpetuated his enthusiasm and interest in hematology after the spark had been kindled Many years ago Doctor Downey was quite interested in comparative neurology and started to develop such a course in the Department of Animal Biology at the University of Minnesota At that time the medical men at Minnesota raised such strong objections to the invasion of the field of neurology by a nonmedical man that Doctor Downey relinquished his newly developed course He then started to give a seminar on the Biology of Blood Cells Little did the medical men of that early period at Minnesota suspect that this seminar was to develop later into a full fledged course in hematology a subject which today is claimed by some as the private property of internal medicine The original course in hematology placed considerable emphasis on the comparative aspect of hematology In later years as suitable material was collected more emphasis was placed upon human pathologic material

Since his retirement in 1946 Doctor Downey has spent two three month sessions (1947 1949) at the Mayo Clinic giving lectures and working with the staff and with fellows specializing in h matology His cytologic study of the megaloblast normoblast problem was an outgrowth of these two sessions He

is still doing a little research when his health permits and at the present time is working on a lymphocyte problem

In closing I wish to quote a paragraph from one of Doctor Downey s recent letters to me It is a crime that I cannot attend a meeting at which I am to be honored However I am not out of the woods yet and if I went to New York it would be strictly against the advice of my physician They don t want me to do anything that will upset their good work Recovery has been good during the past year but is not yet complete so I still have to be careful about what I do

I am sure we all regret that Doctor Hal Downey is unable to be present to hear the part he and his students had in work which led to some of the things discussed at this Conference held in his honor

References

1 Downey H 1909 Folia Haematol 8 415–466
2 Downey H & F Weidenreich 1912 Arch mikroskop Anat 80(Abt 1) 306
3 Downey H 1913 Folia Haematol 15 25
4 Downey H *et al* 1930 Folia Haematol 41 55
5 Downey H *et al* 1939 Folia Haematol 62 1
6 Ehrich W & T Harris 1942 J Exptl Med 76 335
7 Dougherty T F J H Chase & A White 1944 Proc Soc Exptl Biol Med 57 295
8 Mandelbaum F S & H Downey 1916 Folia Haematol 20 139

Part I Introduction to the Leukocytes

LYMPHOCYTES AND PLASMA CELLS

By R. Dorothy Sundberg

Department of Anatomy, University of Minnesota Medical School, Minneapolis, Minn.

Lymphocytes and plasma cells often occur together in lymphatic tissues, in the bone marrow, in connective tissues, and in the blood. Immature forms of both lymphocytes and plasma cells show morphologic similarities which have been appreciated for many years, and even mature appearing lymphocytes seem capable of transformation to plasma cells. The intimate relationship between the two cell types has made it difficult to organize them into clear-cut cell series comparable to the cells of the granulocytic series. Recent experimental data provide evidence which helps both to separate and unite lymphocytes and plasma cells.

Lymphocytes

The problem of lymphocytogenesis has been of interest to Downey[1-8] for many years. Downey and Weidenreich (1912)[1] working with sections of lymph nodes and spleens of animals delineated many fundamentals which have withstood the tests of time and new techniques. Downey and Weidenreich[1] showed that lymphocytes developed from reticular cells, that mitoses were most numerous in large and medium sized lymphocytes, and that mitoses could occur in *any* portion of nodes but were most numerous in germinal centers. In addition to reticular cells and lymphocytes of all sizes, they illustrated plasma cells as one component of germinal centers. Macrophages showing nuclei comparable either to those of reticular cells or of small lymphocytes were also shown. The transformation of lymphocytes (of any size) to granulocytes was clearly depicted. The shedding of small bits of cytoplasm with the resultant hyaline bodies, a phenomenon of great current interest, can also be seen in their figures. In many of their figures it can be ascertained that lymph within sinuses or vessels can contain lymphocytes of all sizes (some in mitosis), reticular cells, and macrophages, as well as hyaline bodies.

Much of the early work has been reviewed elsewhere.[8,10] Here a re-evaluation of terminologies and their reasons for existence seems necessary. With the use of imprint and dry smear techniques and the staining of these preparations with May-Grunwald or Giemsa stains[8,10] we adopted a descriptive terminology for the large and medium sized lymphocytes of the nodes. The latter two-cell types are not seen in normal blood, and the terms large and medium sized lymphocytes are, therefore, confusing. The terminologies may be compared as shown in TABLE 1.

All of these cell types are capable of mitoses, but mitoses are relatively rare in the small lymphocytes of the nodes. I did not encounter mitoses in cells

is still doing a little research when his health permits and at the present time is working on a lymphocyte problem

In closing I wish to quote a paragraph from one of Doctor Downey s recent letters to me It is a crime that I cannot attend a meeting at which I am to be honored However I am not out of the woods yet and if I went to New York it would be strictly against the advice of my physician They don t want me to do anything that will upset their good work Recovery has been good during the past year but is not yet complete so I still have to be careful about what I do

I am sure we all regret that Doctor Hal Downey is unable to be present to hear the part he and his students had in work which led to some of the things discussed at this Conference held in his honor

References

1 Downey H 1909 Folia Haematol **8** 415–466
2 Downey H & F Weidenreich 1912 Arch mikroskop Anat **80**(Abt 1) 306
3 Downey H 1913 Folia Haematol **15** 25
4 Downey H *et al* 1930 Folia Haematol **41** 55
5 Downey H *et al* 1939 Folia Haematol **62** 1
6 Ehrich W & T Harris 1942 J Exptl Med **76** 335
7 Dougherty T F J H Chase & A White 1944 Proc Soc Exptl Biol Med **57** 295
8 Mandelbaum F S & H Downey 1916 Folia Haematol **20** 139

phomatous marrow, but I am unable to judge whether or not the lymphocytes in the marrow have come from the blood as the experiments of Farr suggest [21, 22]

Yoffey [23] in his clear cut paper on the mammalian lymphocyte reviewed previous work which indicated that the bone marrow contains large enough numbers of lymphocytes to indicate that it may be considered one of the largest collections of lymphocytes in the body He emphasized the facts that perivascular nodules of lymphocytes are relatively numerous and that these nodules generally lacked germinal centers and mitoses This is overwhelmingly true in my material which consists of marrow from approximately 3000 patients with section material from well over 50 per cent of the specimens obtained * I have referred to the collections of lymphocytes in the marrow as perivascular lymphocytic aggregates rather than nodules because they generally lack germinal centers or mitoses visible in sections When germinal centers are present as for example in allergic inflammation mitoses are seen in sections and reticular lymphocytes are present in marrow films Although one may find cells which could be lymphoblasts rather than myeloblasts immature lymphocytes and mitoses in cells believed to be lymphocytes in films of marrow and yet see no aggregates of lymphocytes in sections generally when one does find reticular lymphocytes in films of the marrow there may be true follicles with germinal centers in the sections [19] or there may be various types of granulomatous lesions [24, 25]

Presence of Hematopoietic Reticular Cells and Reticular Lymphocytes in the Blood

Hematopoietic reticular cells and reticular lymphocytes comparable to those seen in imprints or dry films of lymph nodes spleen or bone marrow are found in the blood under various conditions

Hematopoietic reticular cells are found in the blood in leukemic reticuloendotheliosis where evidence of their differentiation to any type of blood cell may be present and in some cases of chronic lymphatic leukemia where small numbers of hematopoietic reticular cells and reticular lymphocytes are seen Very occasional hematopoietic reticular cells may be found in the blood in any type of leukemia in spite of the fact that the dominant cells might be lymphoblasts myeloblasts immature monocytes immature granulocytes or developing red cells and megakaryocytes I have seen very occasional cells with the classic morphologic features of hematopoietic reticular cells in occasional cases of Hodgkin s disease and of septicemia One very unusual case of infectious mononucleosis in a child showed in its initial blood picture histiocytes reticular cells in syncytial arrangement and hematopoietic reticular cells as well as reticular lymphocytes and leukocytoid lymphocytes

Reticular lymphocytes may be found in the blood in leukemias most commonly in leukemic reticuloendotheliosis and in some cases of chronic lymphatic leukemia However when only a small percentage of reticular lymphocytes is seen in the blood they are ordinarily associated with some type of lymphocytic reaction

The most outstanding lymphocytic reaction is infectious mononucleosis but

* Thi [illegible] se t [illegible] t m t I h ld t h d st m t [illegible] m g th umbe f lymphocyt gg g t

TABLE 1

Sections of Lymphatic Tissue	*Imprints of Lymphatic Tissue*
Primitive reticular cells	Primitive reticular cells
↓	↓
Giant[11] and large lymphocytes (hemocytoblasts)	Hematopoietic reticular cells
↓	↓
Medium sized lymphocytes	Reticular lymphocytes
↓	↓
Small lymphocytes	Large medium sized and small lymphocytes

the size of ordinary lymphocytes[10] but Trowell (1951)[1] has recently provided illustrations of small lymphocytes in mitosis from his cultures of lymph nodes

Lymphoblasts

We have not used the term lymphoblast with respect to normal lymphocytogenesis in nodes because the original morphologic concept of the lymphoblast was a cell indistinguishable from the myeloblast[5, 8, 10, 13, 17] According to the Downey terminology lymphoblasts are numerous in subacute and acute lymphatic leukemias[5, 18] but they are extremely sparse or absent in normal or benign hyperplastic lymph nodes[8, 10]

Immature Lymphocytes

There is a cell with a narrow rim of pale or deep blue usually homogeneous cytoplasm and a nucleus which may have visible nucleoli and delicate chromatin but chromatin which is sufficiently coarse clumped or smudged to indicate that it is a precursor of lymphocytes which provides difficulty in morphologic interpretation because of its remarkable similarity to the lymphoblast This cell * called an immature lymphocyte[5, 10, 17] is comparable to the prolymphocyte of Ferrata[19] It occurs in both lymph nodes and bone marrow[10, 17] and is seen in the blood of normal infants and children as well as in lymphocytic reactions and all types of lymphatic leukemias[9] In the bone marrow where these immature lymphocytes are seen in association with lymphocytes and blast forms which could be *either* lymphoblasts or myeloblasts one can conclude that there exists a developmental series which proceeds from lymphoblast to immature lymphocyte to lymphocyte or an anaplastic series which proceeds from lymphocyte to immature lymphocyte to lymphoblast (or myeloblast)[17]

Although I have found no mitoses in cells with the size range of ordinary lymphocytes in imprints of nonleukemic lymph nodes[8, 10] mitoses in relatively small cells with pale blue or colorless hyaline cytoplasm are not uncommon in films of nonleukemic bone marrow from patients in all age ranges[9] I interpret the latter finding to mean that lymphocytes multiply in nonleukemic nonlym

I o labo t ry w f t ly r f r to th s cell th cold bl st s pposed t th h t l la t —th lymph bla t f s b ut a d cut lymph t leukem

phomatous marrow but I am unable to judge whether or not the lymphocytes in the marrow have come from the blood as the experiments of Jarr suggest [21, 22]

Yoffey [41] in his clear cut paper on the mammalian lymphocyte reviewed previous work which indicated that the bone marrow contains large enough numbers of lymphocytes to indicate that it may be considered one of the largest collections of lymphocytes in the body He emphasized the facts that perivascular nodules of lymphocytes are relatively numerous and that these nodules generally lacked germinal centers and mitoses This is overwhelmingly true in my material which consists of marrow from approximately 3000 patients with section material from well over 50 per cent of the specimens obtained * I have referred to the collections of lymphocytes in the marrow as perivascular lymphocytic aggregates rather than nodules because they generally lack germinal centers or mitoses visible in sections When germinal centers are present as for example in allergic inflammation mitoses are seen in sections and reticular lymphocytes are present in marrow films Although one may find cells which could be lymphoblasts rather than myeloblasts immature lymphocytes and mitoses in cells believed to be lymphocytes in films of marrow and yet see no aggregates of lymphocytes in sections generally when one does find reticular lymphocytes in films of the marrow there may be true follicles with germinal centers in the sections [19] or there may be various types of granulomatous lesions [4, 25]

Presence of Hematopoietic Reticular Cells and Reticular Lymphocytes in the Blood

Hematopoietic reticular cells and reticular lymphocytes comparable to those seen in imprints or dry films of lymph nodes spleen or bone marrow are found in the blood under various conditions

Hematopoietic reticular cells are found in the blood in leukemic reticuloendotheliosis where evidence of their differentiation to any type of blood cell may be present and in some cases of chronic lymphatic leukemia where small numbers of hematopoietic reticular cells and reticular lymphocytes are seen Very occasional hematopoietic reticular cells may be found in the blood in any type of leukemia in spite of the fact that the dominant cells might be lymphoblasts myeloblasts immature monocytes immature granulocytes or developing red cells and megakaryocytes I have seen very occasional cells with the classic morphologic features of hematopoietic reticular cells in occasional cases of Hodgkin s disease and of septicemia One very unusual case of infectious mononucleosis in a child showed in its initial blood picture histiocytes reticular cells in syncytial arrangement and hematopoietic reticular cells as well as reticular lymphocytes and leukocytoid lymphocytes

Reticular lymphocytes may be found in the blood in leukemias most commonly in leukemic reticuloendotheliosis and in some cases of chronic lymphatic leukemia However when only a small percentage of reticular lymphocytes is seen in the blood they are ordinarily associated with some type of lymphocytic reaction

The most outstanding lymphocytic reaction is infectious mononucleosis but

* Th serv t t m t I should th za d e t m t i g th umbe f lymphocyt
gg g t

TABLE 1

Sections of Lymphatic Tissue	*Imprints of Lymphatic Tissue*
Primitive reticular cells	Primitive reticular cells
↓	↓
Giant[11] and large lymphocytes (hemocytoblasts)	Hematopoietic reticular cells
↓	↓
Medium sized lymphocytes	Reticular lymphocytes
↓	↓
Small lymphocytes	Large, medium sized and small lymphocytes

the size of ordinary lymphocytes[10] but Trowell (1951)[1] has recently provided illustrations of small lymphocytes in mitosis from his cultures of lymph nodes

Lymphoblasts

We have not used the term lymphoblast with respect to normal lymphocytogenesis in nodes because the original morphologic concept of the lymphoblast was a cell indistinguishable from the myeloblast[5, 8, 10, 13, 17] According to the Downey terminology lymphoblasts are numerous in subacute and acute lymphatic leukemias[5, 18] but they are extremely sparse or absent in normal or benign hyperplastic lymph nodes[8, 10]

Immature Lymphocytes

There is a cell with a narrow rim of pale or deep blue usually homogeneous cytoplasm and a nucleus which may have visible nucleoli and delicate chromatin but chromatin which is sufficiently coarse clumped or smudged to indicate that it is a precursor of lymphocytes which provides difficulty in morphologic interpretation because of its remarkable similarity to the lymphoblast This cell * called an immature lymphocyte[5, 10, 17] is comparable to the prolymphocyte of Ferrata[19] It occurs in both lymph nodes and bone marrow[10, 17] and is seen in the blood of normal infants and children as well as in lymphocytic reactions and all types of lymphatic leukemias[20] In the bone marrow where these immature lymphocytes are seen in association with lymphocytes and blast forms which could be *either* lymphoblasts or myeloblasts one can conclude that there exists a developmental series which proceeds from lymphoblast to 'immature lymphocyte to lymphocyte or an anaplastic series which proceeds from lymphocyte to immature lymphocyte to lymphoblast (or myeloblast)[17]

Although I have found no mitoses in cells with the size range of ordinary lymphocytes in imprints of nonleukemic lymph nodes[8, 10] mitoses in relatively small cells with pale blue or colorless hyaline cytoplasm are not uncommon in films of nonleukemic bone marrow from patients in all age ranges[20] I interpret the latter finding to mean that *lymphocytes multiply in nonleukemic nonlym*

I l bo tory we f et ly f to th ell th ld bl t s pposed t th l t bl t —th lymphobla t f s b cut d cute lymph t leukem

of origin and evidence that plasma cells may be derived from all types of lymphocytes (section material) perivascular cells mesothelial cells and their wandering derivatives macrophages and fibroblasts was presented by Downey in 1911[41] At that time controversies regarding the nature and possible functions of plasma cells were also prominent Downey s opinions regarding plasma cells were presented as follows

As modern investigations tend to show that the various types of cells found in the connective tissue (including lymphocytes and mesothelial cells but excluding granulocytes) are merely the morphological expression of varying functional conditions of the same species of cells it is not surprising that plasma cells should be developed from all of them

Plasma cells then are not new cells They represent a special condition of various cells which themselves are only special forms of a mesenchymatous element (Pappenheim) [41]

More recent studies have shown that the remarkable increase in plasma cells in the blood in German measles is preceded by a sharp increase in remarkably basophilic cells with reticular nuclei in lymph nodes[45 46] These cells have been called plasmablasts by Moeschlin [45 46] who considered them distinguishable from the precursors of lymphocytes In 1946 Moeschlin[47] ascribed the plasmacytosis of infectious hepatitis to an initial increase in plasmablasts in the spleen In German measles he claimed there was no similar transformation of reticular cells in the marrow and no increase in serum proteins He agreed with Lanolt that there was no plasmacytosis in the marrow in infectious hepatitis comparable to that seen in the spleen These studies were offered as evidence that there is a lymphatic and a myeloid type of plasma cell[45 46 47]

Plasma cells which have nuclei with reticular characteristics are found in small numbers in the bone marrow under normal conditions These cells are generally *perivascular* in location and they usually differ from Moeschlin s plasmablasts by having less discrete cell bodies less basophilic cytoplasm and often smaller nuclei Their distribution suggests that they may be derived from the perivascular cells or from cells which have emigrated from the blood vessels The youngest of these cells have been called plasmazellulare reticulum cells by Rohr[48] and this terminology was also used by Kolouch[49] and by Kolouch Good and Campbell[50] Immature plasma cells comparable to Moeschlin s plasmablasts are less common in bone marrow but they are often found in marrows which contain perivascular aggregates of lymphocytes lymphocytic follicles or various types of granulomatous lesions [0] Either the plasmazellulare reticular cells of Rohr or the plasmablasts of Moeschlin appear to differentiate to plasma cells with the characteristic morphology of the Marschalko type of plasma cell[40] although there is a wide variation in the presence of the Hof and the amount of basophilic cytoplasm Some of the plasma cells derived from lymphocytes are much smaller than those usually encountered in the marrow and many lack the characteristic Hof

One facet of the morphology of plasma cells which has been of interest to me is the presence of occasional colorless to pale pink granules in their perinuclear cytoplasm These granules seem more similar to the granules seen in lym

variable degrees of lymphocytic reaction (reticular lymphocytes and leukocytoid lymphocytes) may occur in many conditions including brucellosis some hemolytic anemias and conditions associated with pronounced plasmacytosis and/or eosinophilia Of some interest is the fact that in acute and subacute lymphatic leukemias in partial or complete hematologic remission (following A Methopterin and/or cortisone therapy) lymphoblasts may not be present in the blood and may be too sparse in the marrow for diagnosis, but a minimal peripheral lymphocytosis may persist and reticular lymphocytes may be found

Functions of Lymphocytes

For many years the functions of the lymphocytes have been assumed to be as numerous as their developmental potentialities Lymphocytes* have been shown to be capable of transformation to granulocytes[1, 27, 31] monocytes[3, 35] macrophages[11, 3, 40] epithelioid cells[3, 33, 35, 39] giant cells[39] fibroblasts[32] and plasma cells[3, 40, 41] Maximow (1907)[4] offered evidence suggesting the transformation of lymphocytes to erythroblasts in his classic experiment on myeloid metaplasia in the kidney Much of the evidence for these transformations is based on tissue culture experiments[43] Kolouch provided a convincing demonstration of the transformation of lymphocytes to macrophages identical with those derived from the tissue in a serial study of the inflammatory response in loose connective tissue[38] Berman used a technique comparable to the imprint technique for tissue cultures of lymphocytes and showed the transformation of lymphocytes to large intensely basophilic cells (polyblasts) which ultimately formed giant cells[39] These polyblasts were remarkably similar to some of the most basophilic reticular lymphocytes in imprints of nodes[20] Downey[36] showed the transformation of lymphocytes to phagocytes within vessels and Rebuck[40] has demonstrated the transformation of lymphocytes to macrophages many times using his special window technique

Plasma Cells

Plasma cells are present in lymphocytic tissues connective tissues and also in the bone marrow Plasma cells and their precursors may be seen in the blood in large numbers in serum sickness[44] German measles[45, 46] and infectious hepatitis[47] In these conditions there is often an associated lymphocytic reaction with some leukocytoid changes in lymphocytes and occasional reticular lymphocytes In German measles particularly one may find both plasmablasts and reticular lymphocytes in the blood films † Plasma cells have also been seen in the blood in smaller numbers many conditions including infectious mononucleosis and other lymphocytic reactions many conditions associated with eosinophilia some hemolytic anemias and septicemias agranulocytosis and aplastic anemia Plasma cells and their precursors are found in the blood in plasma cell leukemia and in variable numbers in multiple myeloma and leukemic reticuloendotheliosis[6]

Immature plasma cells show morphologic features characteristic of their cell

* I some f these t th lymphocytes w la g d med m d ll w th b so h l cyt pla m wh h w ld be mpa bl t h m t po t t la ll t t la l mphocyt

† Th r ss mpt th t plasmabla t T k ll b t p b bly t l lymph yte h lso be r ga ded s T k ll

of origin and evidence that plasma cells may be derived from all types of lymphocytes (section material) perivascular cells mesothelial cells and their wandering derivatives macrophages and fibroblasts was presented by Downey in 1911[41] At that time controversies regarding the nature and possible functions of plasma cells were also prominent Downey s opinions regarding plasma cells were presented as follows

As modern investigations tend to show that the various types of cells found in the connective tissue (including lymphocytes and mesothelial cells but excluding granulocytes) are merely the morphological expression of varying functional conditions of the same species of cells it is not surprising that plasma cells should be developed *from all of them*

Plasma cells then are not new cells They represent a special condition of various cells which themselves are only special forms of a mesenchymatous element (Pappenheim) [41]

More recent studies have shown that the remarkable increase in plasma cells in the blood in German measles is preceded by a sharp increase in remarkably basophilic cells with reticular nuclei in lymph nodes[45 46] These cells have been called *plasmablasts* by Moeschlin [45 46] who considered them distinguishable from the precursors of lymphocytes In 1946 Moeschlin[47] ascribed the plasmacytosis of infectious hepatitis to an initial increase in plasmablasts in the spleen In German measles he claimed there was no similar transformation of reticular cells in the marrow and no increase in serum proteins He agreed with Lanolt that there was no plasmacytosis in the marrow in infectious hepatitis comparable to that seen in the spleen These studies were offered as evidence that there is a lymphatic and a myeloid type of plasma cell[45 46 47]

Plasma cells which have nuclei with reticular characteristics are found in small numbers in the bone marrow under normal conditions These cells are generally *perivascular* in location and they usually differ from Moeschlin s plasmablasts by having less discrete cell bodies less basophilic cytoplasm and often smaller nuclei Their distribution suggests that they may be derived from the perivascular cells or from cells which have emigrated from the blood vessels The youngest of these cells have been called *plasmazellulare reticulum* cells by Rohr[48] and this terminology was also used by Kolouch[49] and by Kolouch Good and Campbell[50] Immature plasma cells comparable to Moeschlin s plasmablasts are less common in bone marrow but they are often found in marrows which contain perivascular aggregates of lymphocytes lymphocytic follicles or various types of granulomatous lesions [8] Either the plasmazellulare reticular cells of Rohr or the plasmablasts of Moeschlin appear to differentiate to plasma cells with the characteristic morphology of the Marschalko type of plasma cell[40] although there is a wide variation in the presence of the Hof and the amount of basophilic cytoplasm Some of the plasma cells derived from lymphocytes are much smaller than those usually encountered in the marrow and many lack the characteristic Hof

One facet of the morphology of plasma cells which has been of interest to me is the presence of occasional colorless to pale pink granules in their perinuclear cytoplasm These granules seem more similar to the granules seen in lym

phoblasts (leukemia, lymphosarcoma) than to those granules which are clearly relatable to the formation of Russel s fuchsin bodies [0]

The function of plasma cells has been more obscure Downey (1911)[41] early pointed to their granuloplasm (the ribonucleic acid bearing portion of their cytoplasm) as 'the morphologic expression of a special secretory activity Downey also described the development of mast granules in the cytoplasm of plasma cells resulting in so called plasma mast cells (Krompecher Marschalko Weishaupt Pappenheim Schridde)'[41] Although a case of plasma cell leukemia in which plasma cells have shown remarkable phagocytosis of erythrocytes has been described[51] and hemosiderin[5] [53] and various crystals[20] [54] [56] have been seen in the cytoplasma of plasma cells of the marrow mature plasma cells are apparently not remarkably phagocytic Of some interest is the fact that Goodpasture[57] found typhoid bacilli in immature plasma cells

Michels (1931)[58] in addition to analyzing and organizing the vast literature on the subject of plasma cells included some of the speculations on the function of these cells He pointed out that Huebschmann (1913) regarded plasma cells as elements capable of elaborating a defense (antitoxic) substance and that Klein (1914) and Arneth (1920) maintained that plasma cells especially those of chronic disturbances (paralysis meningitis) are not degenerative cell forms but functional states of lymphocytes which through local toxic activation are intimately related to immunization processes

Currently the problem of the cell responsible for antibody production has provided motivation to make new attempts to identify and understand lymphocytes and plasma cells

Cells Responsible for Production of Antibodies

As early as 1925 Bunting[59] postulated that lymphocytes manufactured antitoxins McMaster and Hudeck (1935)[60] demonstrated agglutinins in regional lymph nodes of mice about one week after the injection of antigens intradermally Ehrich and Harris (1942)[61] injected typhoid vaccine and erythrocytes into the foot pads of rabbits and then studied the afferent lymph the popliteal lymph node and the efferent lymph as well as the blood with reference to antibody titer Antibodies began to appear in the lymph two to four days after injection of the antigen and reached their highest titer after six days The latter titer was higher than that in the blood The peak titer was preceded by an outpouring of lymphocytes from the node The node underwent initial diffuse hyperplasia and secondarily large germinal centers appeared The germinal centers appeared later than the rise in antibody titer The experiments were interpreted as pointing to the lymphocyte as a factor in the formation of antibodies Beginning in 1943 Dougherty and White and Dougherty White and Chase presented evidence that was interpreted as indicating that lymphocytes carried antibody within their cell bodies[6] Their experiments suggested that adrenal cortical hormones enhanced the antibody titer[63] In mice the decrease of lymphocytes in lymphoid tissue[64] and the concomitant lymphopenia following injection of pituitary adrenotrophic hormone or adrenal

cortical extract were in their time relationships correlated with an increase in serum proteins [66, 67] An anamnestic reaction followed the injection of ACTH or ACE but it did not follow the injection of ACTH in adrenalectomized immunized rabbits and mice or the injection of desoxycorticosterone acetate [68] These data were regarded as establishing the role of pituitary adrenal cortical secretion as the controlling mechanism for the release of antibody from lymphocytes [68] The alterations in lymphoid tissue of mice and rabbits induced by adrenal cortical secretion were described and illustrated by Dougherty and White in 1945 [69] and described and correlated with early experiments in 1947 [70] These consisted in initial degenerative changes persisting through the first six hours characterized by extreme edema pyknosis of medium-sized and small lymphocytes shedding of cytoplasm of lymphocytes and cessation of mitosis Repair changes beginning at about six hours included phagocytosis of nuclear debris and deposition of dark basophilic material in the cytoplasm of fixed reticular cell Recovery changes began at nine hours and included mitoses of lymphocytes and maturation of reticular lymphocytes as well as differentiation of reticular lymphocytes from reticular cells Within 24 hours the lymphocytic tissues of mice resembled those in normal animals except for the thymus which was still depleted of lymphocytes [69] In rabbits degenerative changes recurred 24 hours after hormone injection but recovery occurred by 48 hours ACTH or adrenal cortical hormone injection had little degenerative effect on reticular lymphocytes or on granulopoietic cells [69] The effect of hormones on lymphatic tissue has recently been reviewed by Dougherty [71] The *in vitro* lysis of lymphocytes by lymphocytolytic adrenocortical steroids in tissue culture and in cell suspensions was discussed and Dougherty watched and recorded the lysis of single lymphocytes by means of phase microscopy and interval photographs Lymphocyte budding is produced *in vitro* by both compound F and cortisone Hyaline bodies the cytoplasmic fragments resulting from this budding are thought to be a manifestation of secretion [66]

Transfer of Antibody-Containing Cells

One of the interesting earlier experiments of Dougherty White and Chase was the demonstration of antibodies in malignant lymphocytes [72] A transplanted nonmetastasizing mouse lymphosarcoma was shown to contain a high inhibition titer for an hemolytic toxin of *Staphylococcus aureus* even when antigen was not administered after transplantation and transplants of antibody-containing tumor to nonimmunized mice contained *immune globulin* 13 to 17 days after transplantation. The titer in the tumor was frequently higher than in the normal lymphocytes The tumor cells illustrated in a publication with Gardner and Williams [73] show cells which resemble reticular lymphocytes Here one might point out that some so-called lymphosarcoma cells can have exceedingly basophilic cytoplasm

Wesslen recently (19 ?) showed that lymphocytes from thoracic duct lymph from animals immunized with *S typhi* or horse serum did not contain antibodies but were capable of producing them He claimed that only 5 to 6 per cent of the lymphocytes were large the rest being small He felt there were

phoblasts (leukemia lymphosarcoma) than to those granules which are clearly relatable to the formation of Russel s fuchsin bodies [0]

The function of plasma cells has been more obscure Downey (1911)[41] early pointed to their granuloplasm (the ribonucleic acid bearing portion of their cytoplasm) as 'the morphologic expression of a special secretory activity Downey also described the development of mast granules in the cytoplasm of plasma cells resulting in so called plasma mast cells (Krompecher Mar schalko Weishaupt Pappenheim Schridde) [41] Although a case of plasma cell leukemia in which plasma cells have shown remarkable phagocytosis of erythrocytes has been described [51] and hemosiderin[5] [53] and various crys tals [0] [54] [56] have been seen in the cytoplasma of plasma cells of the marrow mature plasma cells are apparently not remarkably phagocytic Of some interest is the fact that Goodpasture[57] found typhoid bacilli in immature plasma cells

Michels (1931) [58] in addition to analyzing and organizing the vast literature on the subject of plasma cells included some of the speculations on the function of these cells He pointed out that Huebschmann (1913) regarded plasma cells as elements capable of elaborating a defense (antitoxic) substance and that Klein (1914) and Arneth (1920) maintained that plasma cells especially those of chronic disturbances (paralysis meningitis) are not degenerative cell forms, but functional states of lymphocytes which through local toxic activation are intimately related to immunization processes

Currently the problem of the cell responsible for antibody production has provided motivation to make new attempts to identify and understand lym phocytes and plasma cells

Cells Responsible for Production of Antibodies

As early as 1925 Bunting[59] postulated that lymphocytes manufactured anti toxins McMaster and Hudeck (1935)[60] demonstrated agglutinins in regional lymph nodes of mice about one week after the injection of antigens intrader mally Ehrich and Harris (1942)[61] injected typhoid vaccine and erythrocytes into the foot pads of rabbits and then studied the afferent lymph the popliteal lymph node and the efferent lymph as well as the blood with reference to anti body titer Antibodies began to appear in the lymph two to four days after injection of the antigen and reached their highest titer after six days The latter titer was higher than that in the blood The peak titer was preceded by an outpouring of lymphocytes from the node The node underwent initial diffuse hyperplasia and secondarily large germinal centers appeared The germinal centers appeared later than the rise in antibody titer The experi ments were interpreted as pointing to the lymphocyte as a factor in the forma tion of antibodies Beginning in 1943 Dougherty and White and Dougherty White and Chase presented evidence that was interpreted as indicating that lymphocytes carried antibody within their cell bodies [6] Their experiments suggested that adrenal cortical hormones enhanced the antibody titer [63] In mice the decrease of lymphocytes in lymphoid tissue[64] and the concomitant lymphopenia following injection of pituitary adrenotrophic hormone or adrenal

to appear in the sera of recipients in four days. Similar results were obtained with a soluble antigen of the dysentery organisms.

When these experiments are added to those of Dougherty, White and Chase[77] and Wesslen[78] it would certainly appear that cells in lymph nodes, including those presumably destined for circulation, whatever their specific identity, are capable of antibody production. If one is to accept these antibody forming cell as plasma cells or their precursors, one must assume that:

(1) Lymphosarcoma has primary or secondary plasmacellular components.

(2) The thoracic duct lymph cultured by Wesslen contained plasma cells and/or their precursors. These could be the 5 to 6 per cent large lymphocytes which could easily include potential plasma cells, or the more numerous small lymphocytes which can also form plasma cells. Wesslen denied the presence of plasma cell in the lymph.

Neither of the explanations offered seems deniable although they are not the first which would come to mind.

Serum Globulin, Plasma Cells, Antibodies

Since hyperglobulinemia and plasmacytosis show a high degree of correlation (Bing and Plum, 1937)[79] and since antibodies are believed to reside in the globulin fraction of the serum proteins, attempts to link plasma cells with antibody production have been numerous. Early literature is reviewed by Cormsen and Heintzelmann (1941)[50] and Kolouch, Good and Campbell (1947).[80] Kolouch[49] as early as 1938 suggested that there might be a relationship between the transformation of plasmacytic reticulum cells of the marrow to plasma cells and rising antibody titer. Kolouch's studies were stimulated by Downey's (1911)[41] description of a plasmacytosis in the bone marrow of a rabbit with a nonspecific subcutaneous abscess and by use of rabbits sensitized to *Streptococcus viridans*. Later studies of Kolouch, Good and Campbell (1947)[80] suggested that the morphologic differentiation of reticular plasma cell to Marschalkó plasma cells, which took four days after shocking a rabbit sensitized to *Str. viridans* or egg white, was intimately associated with the production of antibodies. Good (1950)[51] described many plasma cells and precursors in acute experimental allergic encephalitis. These were almost completely absent in the acute stages of nonallergic inflammation, but a few plasma cells were found in the inflammatory exudate late in the nonallergic inflammatory cycle. Campbell and Good (1950)[57] made guinea pigs encephalitic by sensitization to homologous brain tissue plus adjuvants, and produced intense plasmacytosis in the brain, spleen, and bone marrow. In these experiments they found large numbers of plasma cells derived from lymphocytes as well as from reticular cells and concluded that *these cell lines were essentially continuous*. Their Figure 2 shows the theoretical development of plasma cells from reticular cells *with* and *without* an intermediary reticular lymphocyte. They do not postulate transitions of ordinary lymphocytes to plasma cells. Good and Campbell (1950)[53] showed excellent correlation between the numbers of plasma cells (per 5000 nucleated marrow cells) in the bone marrow and the level of serum

no plasma cells in thoracic duct lymph and that one would not expect to find them there His experiment was offered as one in which there was a *pure* lymph cell suspension

Keuning and van der Slikke (1950)[75] found that at the height of antibody production (three to four days) in rabbits immunized by three intravenous injections of paratyphoid B vaccine red pulp material, containing characteristic aggregates of immature plasma cells produced more agglutinin than white pulp in tissue culture They felt their experiments clearly refuted the idea of antibody production by mature lymphocytes However since immature lymphoid cells of the white pulp where no plasma cells are found' apparently produced agglutinin *in vitro* Keuning and van der Slikke thought that lymphoblastic cells of Malpighian corpuscles were probably involved in antibody production to some extent Here it may be commented that it seems unnecessary to exclude plasma cells from germinal centers in that they have been an accepted component of germinal centers for many years [Downey & Weidenreich (1912)[1] Bloom (1938)[54]]

Harris Harris and Farber (1954)[76] have *recently provided further evidence* that viable washed lymph node cells from immunized rabbits show continued ability to form antibodies in a recipient nonimmunized rabbit Antibody appeared in the recipient s serum on the first day after transfer (sooner than if this phenomenon were ascribed to transfer of antigen) rose in titer on days 2 and 3 and began to decline on day 5 or 7 Antibodies were no longer measurable by the 23rd or 40th day Here as in Wesslen s experiments [68] the evoked antibody titer appeared to bear a relationship to the number of cells transferred When the cells to be transferred were rendered nonviable, they were no longer capable of giving rise to the appearance of antibody in the recipients When the methods used to render the cells nonviable were applied to hyperimmune anti Shigella serum or dysentery bacilli the actions of the serum and the bacilli were not destroyed

Harris Harris and Farber (1954)[77] also showed that when the antigen was injected in the foot pads the antibody titer demonstrable in the recipient of washed cells from the popliteal node was high but that from washed splenic cells was low or not demonstrable Conversely if the antigen was injected intravenously the transferred washed splenic cells produced a high antibody titer in the recipient rabbit but the cells from the popliteal node gave rise to a much lower titer Neither of these routes of immunization of the donor rabbits apparently caused changes in the cells of the mesenteric lymph nodes as washed cells from the latter nodes did not produce an antibody titer in the recipient animal If four antigens were used four antibodies could be transferred

Harris Harris and Farber (1954)[78] found that donor cells from regional nodes of rabbits which had received antigen 10 minutes previously were capable of causing the appearance of antibody in recipient rabbits The recipient rabbits had previously received whole body X radiation Heated similar cells were not effective Lymph node cells incubated with dysentery organisms at 37° C *in vitro* washed and injected into X radiated rabbits caused agglutinins

the cytoplasm of various stages of immature plasma cells from the red pulp of spleens of rabbits sensitized and reinjected with TAB vaccine These granules were most numerous on the fifth day immediately prior to the appearance of antibodies in the blood The granules were thought to be comparable to similar granules in myeloma cells Neither was visible with ordinary staining methods

Kunkel Slater and Good (1951)[93] demonstrated that the majority of myeloma proteins are immunologically related to a portion of normal gamma globulin This demonstration added further evidence for a role by plasma cells in the production of at least a component of normal gamma globulin

Coons Leduc and Connolly (1953)[94] using a fluorescein labelled antibody technique detected antibody in the regional node four days after the first subcutaneous injection of antigen The antibody was found in occasional cells scattered singly in the medullary cords and around the periphery of but only rarely within the lymphoid follicles The cells had large nuclei and a thin rim of cytoplasm Traces of antibody were seen in the nucleus and high concentrations were found in the cytoplasm At six to eight days the number of cells included was not increased but the cells had become typical plasma cells In the secondary response antibody was first detectable on the second day in younger cells but their development followed the same course More cells were involved and they were grouped in colonies suggesting that 'the descendants of some scattered primitive cells were now all engaged in antibody synthesis The data are regarded as confirming the conclusion of others that the plasma cell is the major source of antibody under these conditions although they do not exclude smaller contributions by other cell types

Good (1954)[95] studied two patients with no measurable gamma globulin no evidence of tissue or serum antibody and no isoagglutinins against heterologous blood groups Intense antigenic stimulation did not evoke an antibody titer or cause the appearance of gamma globulin in the plasma A third patient is now added to this group [96] Plasma cells are virtually absent from the marrow in these patients and intense antigenic stimulation does not produce plasmacytosis of the marrow or nodes as it does in normal subjects I have had the opportunity of seeing this material There is a remarkable plasmacellular reaction in the control nodes after antigenic stimulation I have seen no plasma cells in a similar node from the third patient I have found extremely occasional plasma cells in the marrow of two of these patients but I surely agree that plasma cells are conspicuous by their virtual absence

Craig Gitlin and Jewett (1954)[97] reported similar findings using the fluorescent antibody technique In nodes from normal children who had received combined DPT and typhoid paratyphoid vaccine four days prior to removal *of the nodes well defined follicles with active germinal centers were seen and* the medullary cords were increased in prominence Plasma and preplasma cells appeared in both the cortex and medulla Gamma globulin was seen in various cells of the interstitial fluid and pertussis antigen could easily be demonstrated in the reticuloendothelial cells Specific antibodies against diphtheria and tetanus toxins were found in cells of the plasma cell series In nodes from children with agammaglobulinemia the cortex was narrower follicles

gamma globulin in normal children and in children in various stages of activity *of rheumatic fever chorea and streptococcal pharyngitis*

Bjørneboe Gormsen and Lundquist (1947)[84] added further weight to early (1941[85] 1943[87] 1943[83]) experiments which suggested that with strong immunization there was hyperglobulinemia and a pronounced plasma cell formation in all organs apparently proportional to the concentration of antibody protein Because a constant finding was intense perivascular plasmacytosis in the adipose tissue of the renal sinus this tissue (90 per cent plasma cells and 10 per cent lymphocytes) was extracted for antibody content Since it was found to have essentially more antibody than the extracts of any other organs from the rabbits immunized with a mixture of 8 pneumococcus types Bjørneboe, Gormsen & Lundquist[84] advanced the hypothesis that antibodies are produced by plasma cells

In 1944 Thorell and Wissing[88] found the myeloma cell possessed the characteristics of a cell which functions to produce new proteins Bing Fagraeus and Thorell[89] using ultra violet microscopy and the Feulgen reaction, demonstrated that immature plasma cells contain an abundance of ribose nucleotides indicating active protein synthesis while more mature plasma cells contain less ribose nucleotides Fagraeus (1948)[90] using horse serum and *S typhi* as antigens produced anaphylactic shock in rabbits and followed the cellular reaction in the spleen and the antibody content in the serum At two to three days she described and illustrated immature cells (transitional cells) in the red pulp in the reaction centers and at the marginal zone of the Malpighian corpuscles At three to five days as the antibody titers in the serum were beginning to rise the predominant cell in the spleen was somewhat smaller (immature plasma cell) At five to eight days at the peak of the antibody titers in the serum the predominant cell was the plasma cell Tissue cultures of small portions of red pulp (rich in plasma cells) and Malpighian bodies (rich in lymphocytes) from the experimental animals revealed that the antibody units per gram of tissue were consistently *significantly higher in the red than in the white pulp The amount* of antibody production was greater at four to five days when transitional and immature plasma cells predominated than at seven to ten days when mature plasma cells were the most numerous Fagraeus concluded that antibodies are formed within reticuloendothelial cells In case of an intense antibody formation a differentiation of these cells into plasma cells takes place Thus the mature plasma cell is to be regarded as the small link in a chain of development a cell which has already passed the state of its greatest functional intensity'[90]

Reiss Mertens and Ehrich (1950)[91] demonstrated that certain lymphoid cells of antibody forming lymph nodes agglutinate the bacteria with which the animals were immunized on their surface *in vitro* Neutrophils monocytes macrophages immature lymphocytes and typical small lymphocytes did not agglutinate the bacteria The identifiable agglutinating cells belonged to the plasma cell series Some small cells believed to be close to a mitotic division showed remarkable agglutinating power The most pronounced reaction occurred at five to seven days

Moeschlin (1951)[92] showed by means of phase microscopy dark granules in

Bibliography

1 Downey H & F Weidenreich 1912 Arch mikroskop Anat Entwicklungsmech 80 306
2 Downey H 1912 Trans Minn Path Soc 1 91
3 Thiel G A & H Downey 1921 Am J Anat 38 2 9
4 Downey H & C A McKinlay 1923 Arch Internal Med 32 82
5 Stasney J & H Downey 1935 Am J Path 11 113
6 Downey H & J Stasney 1936 Folia Haematol 54 417
Downey H 1935 Anat Record (Suppl) 61 14
8 Sundberg R D & H Downey 1942 Am J Anat 70 455
9 Sundberg D 1941 Detroit Med News Educ Issue 32 29
10 Sundberg R D 1947 J Lab Clin Med 32 777
11 Taliaferro W H & H W Mulligan 1937 Indian Med Research Mem 29 1
12 Trowell O A 1952 Exptl Cell Research 3 79
13 Downey H 1924 Arch Internal Med 33 301
14 Downey H 1927 Folia Haematol 34 65
15 Downey H 1932 Special Cytology 651 2nd ed E V Cowdry Ed Hoeber New York N Y
16 Downey H 1938 Handbook of Hematology 1963 H Downey Ed Hoeber New York N Y
17 Sundberg R D 1943 Thesis Univ Minnesota
18 Sundberg R D 1946 Staff Meeting Bull Hosp Univ Minn 17(26) 389
19 Ferrata A 1933 Le Emopatie 2nd ed Vol I Soc Ed Lib Milano
20 *Sundberg R D Unpublished observations*
21 Farr R S 1946 Anat Record 94 460
22 Farr R S 1950 Anat Record 106 28
23 Yoffey J M 1950 Biol Revs Cambridge Phil Soc 25 314
24 Sundberg R D & W W Spink 1947 Blood Supp No 1 7
25 Hovde R & R D Sundberg 1950 Blood 5 209
26 Downey H 1938 Handbook of Hematology 12 3 H Downey Ed Hoeber New York N Y
27 Dominici H 1909 Folia Haematol 8 97
28 Maximow A 1923 Arch mikroskop Anat Entwicklungsmech 97 283
29 Maximow A 1923 Arch mikroskop Anat Entwicklungsmech 97 314
30 Bloom W 1926 Folia Haematol 33 122
31 Lang F J 1926 Ztschr mikroskop anat Forsch 4 417
32 Maximow A A & W Bloom 1952 A Textbook of Histology 6th Ed W Bloom Ed Saunders Philadelphia Pa
33 Conway E A 1937 Anat Record 69 487
34 Conway E A 1938 Arch Path 25 200
35 Conway E A 1939 J Infectious Diseases 64 217
36 Downey H 1917 Anat Record 12 429
37 Watson C J 1928 Folia Haematol 37 70
38 Kolouch F 1939 Am J Path 15 413
39 Berman L 1942 Arch Path 33 295
40 Rebuck J W 1951 Gastroenterology 19 644
41 Downey H 1911 Folia Haematol 11 275
42 Maximow A 1907 Beit path Anat u allgem Path 41 122
43 Bloom W 1938 Handbook of Hematology 1470 H Downey Ed Hoeber New York N Y
44 Markoff N 1937 Deut Arch klin Med 180 530
45 Moeschlin S 1940 Helv Med Acta 7 227
46 Moeschlin S 1941 Deut Arch klin Med 187 249
47 Moeschlin S 1946 Gasteroenterologia 71 97
48 Rohr K 1936 Folia Haematol 55 305
49 Kolouch F 1938 Proc Soc Exptl Biol Med 29 147
50 Kolouch F R A Good & B Campbell 1947 J Lab Clin Med 32 749
51 Butterworth C E Jr W B Frommeyer Jr & W H Riser Jr 1953 Blood 8 519
52 Kozewski B J 1952 Blood 7 1182
53 Sundberg R D & H Broman 1954 To be published
54 Bloom W 1938 Handbook of Hematology 1429 H Downey Ed Hoeber New York N Y

were small and ill defined, and germinal centers and preplasma and plasma cells were absent Antigen was easily demonstrated in these nodes but neither gamma globulin nor pecific antibody could be found The conclusion drawn was that in congenital agammaglobulinemia there is a deficiency in the production of the cell forms responsible for the formation of antibody

One piece of evidence which is difficult to organize is that presented by Craddock and Lawrence (1948) [98] These investigators produced a marked lymphopenia lasting 30 to 40 days by exposing rabbits to 250 r whole body roentgen irradiation Injections of antigens eight hours after this exposure elicited a greatly depressed antibody response This depression did not occur if a similar exposure was given after the onset of antibody formation (significant titer at day 4) but before a maximal titer (day 9) was attained although there was thought to be a marked depression of lymphoid tissue as estimated from the peripheral lymphopenia It was suggested that the reserve ources of tissue must be capable of carrying out antibody formation once it has begun even though the major portion of the lymphoid ti sue has been damaged Here one might comment that reticular lymphocytes and plasma cells are less affected by X ray than are mature lymphocytes

The pendulum currently appears to be favoring the precursors of plasma cells as antibody formers Plasma cells and their precursors are present in lymphatic tissue of presumably normal animals and man and here there are also hematopoietic reticular cells and reticular lymphocytes Plasmablasts, hematopoietic reticular cells and reticular lymphocytes are morphologically distinguishable from one another but often their similarities are greater than their differences The most remarkable differences are the intensity and opacity of cytoplasmic basophilia the assumption and retention of an eccentric nucleus and the relatively discrete clumping of the nuclear chromatin of the immature plasma cell Intensely basophilic reticular lymphocytes also occur and it does not seem possible to conclude that they are *necessarily* precursors of plasma cells because transitions between them and lymphocytes are easily found These intensely basophilic reticular lymphocytes are similar to some of the polyblasts[39] in tissue culture experiments however and it would not be surprising to find that these cells could function as either precursors of lymphocytes transitional stages in the formation of macrophages or precursors of plasma cells under the appropriate stimuli

The most recent evidence suggests that precursors of plasma cells contain large amounts of antibody and that when plasma cells and their precursor are virtually absent antibody production is not possible We are still faced with the fact that even with remarkable antibody production plasma cells are often not as numerous as lymphocytes and some type of lymphocytic reaction generally accompanies plasmacytosis Perhaps the controversy will be resolved by some entirely acceptable demonstration of the role lymphocytes play in the production of immature or mature plasma cells

Acknowledgment

I am indebted to Henry Morris for the photomicrographs

PLATES

55 Gormsen H 1942 Thesis Nyt Nordisk Forlag Copenhagen Denmark
56 Sandoz 1950 Planches d Hematologie Planche 24 Sandoz Basle Switzerland
57 Goodpasture W W 1937 Am J Path 13 175
58 Michels N A 1931 Arch Path 11 775
59 Bunting C H 1925 Wisconsin Med J 24 305
60 McMaster P D & S S Hudack 1935 J Exptl Med 61 783
61 Ehrich W E & T N Harris 1942 J Exptl Med 76 335
62 Dougherty T F J H Chase & A White 1944 Proc Soc Exptl Biol Med 54 295
63 Chase J H A White & T F Dougherty 1946 J Immunol 52 101
64 Dougherty T F & A White 1943 Proc Soc Exptl Biol Med 53 132
65 White A & T F Dougherty 1944 Proc Soc Exptl Biol Med 56 26
66 Dougherty T F A White & J H Chase 1944 Proc Soc Exptl Biol Med 56 28
67 White A & T F Dougherty 1945 Endocrinology 36 207
68 Dougherty T F J H Chase & A White 1945 Proc Soc Exptl Biol Med 58 135
69 Dougherty T F & A White 1945 Am J Anat 77 81
70 Dougherty T F & A White 1947 J Lab Clin Med 32 584
71 Dougherty T F 1952 Physiol Revs 32 379
72 Dougherty T F A White & J H Chase 1945 Proc Soc Exptl Biol Med 59 172
73 Gardner W U T F Dougherty & W L Williams 1944 Cancer Research 4 73
74 Wesslén T 1952 Acta Dermato-Venereol 32 265
75 Keuning F J & L B van der Slikke 1950 J Lab Clin Med 36 167
76 Harris S T N Harris & M B Farber 1954 J Immunol 72 148
77 Harris T N, S Harris & M B Farber 1954 J Immunol 72 161
78 Harris T N S Harris & M B Farber 1954 Federation Proc 13 496
79 Bing J & P Plum 1937 Acta Med Scand 92 415
80 Gormsen H & F Heintzelmann 1941 Nord Med 11 2128
81 Good R A 1950 J Neuropath Exptl Neurol 9 78
82 Campbell B & R A Good 1950 Arch Neurol Psychiat 63 298
83 Good R A & B Campbell 1950 Am J Med 9 330
84 Bjørneboe M H Gormsen & F Lundquist 1947 J Immunol 55 121
85 Bjørneboe M & H Gormsen 1941 Nord Med 9 891
86 Bjørneboe M & H Gormsen 1943 Nord Med 19 1155
87 Bjørneboe M & H Gormsen 1943 Acta Path Microbiol Scand 20 649
88 Thorell B & P Wissing 1944 Nord Med 24 1842
89 Bing J A Fagraeus & B Thorell 1945 Acta Physiol Scand 10 282
90 Fagraeus A 1948 Antibody Production in Relation to the Development of Plasma Cells Esselte aktiebolag Stockholm Sweden (also Acta Med Scand 130 suppl 9)
91 Keiss F E Mertens & W E Ehrich 1950 Federation Proc 74 432
92 Moeschlin S J R Pelaez & F Hugentobler 1951 Acta Haematol 6 321
93 Kunkel H G R J Slater & R A Good 1951 Proc Soc Exptl Biol Med 76 190
94 Coons A H F H Leduc & J M Connolly 1953 Federation Proc 12(1) 439
95 Good R A 1954 Soc Ped Res 24th Annual Meeting Pennsylvania 3
96 Good R A 1954 Personal communication
97 Craig J M D Gitlin & T C Jewett 1954 Soc Ped Res 24th Annual Meeting Pennsylvania 4
98 Craddock C G & J S Lawrence 1948 J Immunol 60 241

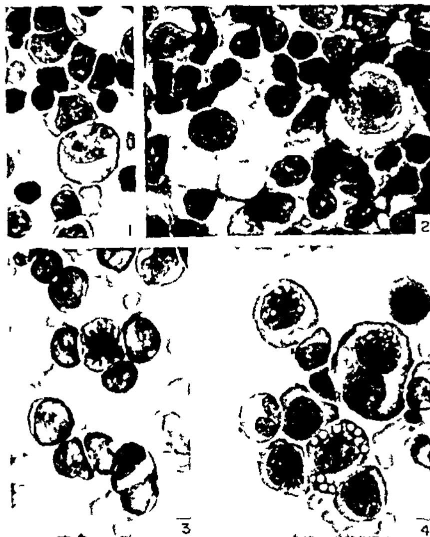
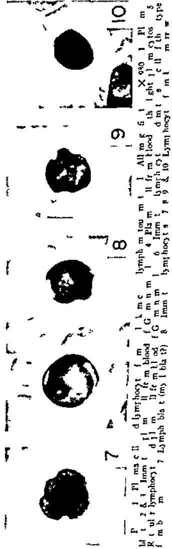
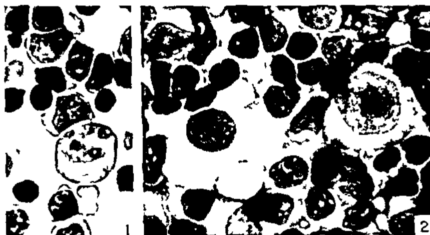
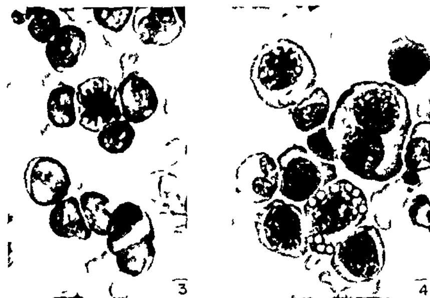


PLATE 2 Dry film nd mp t p pa t f om lymph t l kem 3 m lt p l my l m All m gn fi cat X 784 1 Hem topo t t la ll d lymphocytes f om bo m rrow f h lymph t leuk m 2 Prim e t cula ll d n lly la g hemat po t t l ll d lymphocyt from lymph nod mp t f h on lymph leukem 3 Lymph bla ts mm lymphocyte f tw m t t figu 1 m bo m rr w f subac te lymph t l ekem 4 Plasm ll ou t ge f m t ty tain g d lo g R sel f h bod f om bo m w f m l ple my lom



P 1 Pl ma c ll d lymphocyt f m l k m c lymph m tou m t 1 All m g fi t X 990 1 Pl m bl t 2 & 3 Imm t l m ll fr m blood f G m n m l 4 Pla m ll fr m blood th l ght l m cytos 5 R t ul r lymphocyt d l m ll fr m bl od f G m n m l 6 Imm t lymph cyt d m t s c ll f th type f m b m 7 Lymph bla t (my l bla t?) 8 Imm t lymphocyt s 7 8 9 & 10 Lymphocyt f m l m rr w





P TE 2 Dry film imp t p pa t on f m lymph t l kem l m lt pl my loma All m gn fi t × 84 l llem topo t ul ll d lymphocyte l m bo m ow f h lymph t le k m 2 P m t t l lla d u lly la g h m t po t t l ll d lymphocyt s f m lymph od mp t f h lymphat le kem 3 Lymphobl t mm tu lymphocyt d tw m t t figu es f om bo m rrow f b lymph t l k m 4 Pla m ll t g t m t ty co ta g d lop g R sel f h bod f om bo m ow f m lt pl my lom







PL T 3 Plasm ll f om onmy lom tou bo m row 1 Section f m rrow X [illegible] pe [illegible] 1 plasm ll t th l ft f blood sel 2 Dry film f m row X 560 P n sc la pla m l m l t l plasm ll Bottom those f fou d nea f th edge f film 3 Dry film f m ow X 680 T p m t Plasm ll R type f mm t plasm ll (w th t la ucl chara t ri tic) 4 Dry film f m rr X [illegible] t ulo d th l k ll sh wing s plasma ll t ts pe ph ry

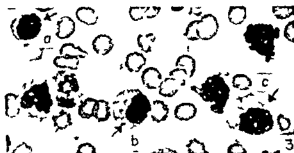
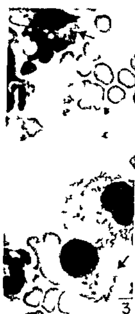


PLATE 4 1 p ad fom t m f gu f X 84 N t pillary w th per sc ll l l m ll
r h l t w h d ff t t f mes th l l ll t f ded ll f n g ze cyt pl m l so-
ph l d l hrom t t tt 2 Dry film f h m sc t fl d X 84 sh g la ge deepl b soph l
l m soth l t ll w th t l l pl m c ll 3 Dry film f sam hum n as t fl d X 680
how g l ss u l zat f y t pl sm d as g d l cy f p l patt r w ll se
ll ze h t format f lymphocyt t phagocyt c ll (m ph g) b d





Pl g 3 Pla m ll f om o my lom t bo m w 1 Sect f m w × 84 t per sc l pla m lls t th l ft f blood sel 2 Dry f lm f m w × 560 F sc la pl m ll m la t th se 1 f d f th dg f film 3 D y f lm f m w × 680 T p mat pl m ll B tt m o typ f mm t pl sm ll (w th t l u l b te st s) 4 Dry film f m × 4 R t loc d th l l ll h g pl sm ll at ts pe ph ry

In the embryo the first evidences of neutrophilic leukocytes are found in the liver in the second lunar month during the hepatic period of hematopoiesis At the 9 cm stage of the human fetus myelocytic cells can be seen developing in the spleen[7] and from approximately the fourth lunar month[8] they are found in the bone marrow The genealogy of the formed elements of the blood has been depicted graphically in 1952[9] by the various proponents of the monophyletic modified neounitarian and polyphyletic theories

In 1951[10] Wintrobe wrote Little is known about these factors (factors which govern maturation formation and release of blood cells into the blood stream) or about those which are concerned with destruction of blood cells The list of these factors is not complete as yet nor has the degree of influence of each singly or in combination been ascertained The splenic factor in maturation has been the subject of investigation on numerous occasions and its importance in radiation in some of the more recent works has been studied Krumbhaar[11] showed that splenectomy can produce a marrow hyperplasia Interestingly enough in the parabiotic animals the leukocytosis which followed failed to develop until the second animal had been splenectomized Jacobson *et al*[12] showed that the shielding of the spleen and intraperitoneal transplantation of spleens after irradiation prevented total marrow aplasia and death after total body X radiation and a more rapid return of hemopoiesis to normal This so-called 'splenic factor' may not be a specific and isolated influence[14] It may be merely a reflection of a protective factor present in all hematopoietic tissue Wright[15] in discussing specific depressions on various cell lines in the treatment of leukemias and cellular defense mechanisms[16] discussed the factors of diet and mentioned the fact that nucleoproteins stimulate myelopoiesis This tendency was shown by Doan[17] and his co-workers in 1928 to be true for the rabbit The release of mature forms as part of a normal rhythm theory was presented first by Sabin *et al*[18, 19] The rhythmic hourly accessions to the peripheral blood stream were believed related to the maturation of myelocytes and was preceded by a shower of nonmotile or dead leukocytes up to 30 per cent The hypothesis was that the dying cell served as an impetus and as mentioned previously products of nucleic acids were a necessary factor for the release of granulocytes Smith and McDowell[20] were able to confirm the rhythmic accession of leukocytes in women but were unable to find large numbers of dead neutrophilic leukocytes in the blood stream to the effect that the neutrophilic curve was influenced by them Carrey and Butler[21, 22] could not confirm either study Koch and Lubbers[23] have recently reported finding leukocytic disintegration forms in the peripheral blood in cases of inflammatory diseases especially in irritative states of the reticuloendothelial system The *appearance of these forms in peripheral blood* was transient and did not correlate with age sex temperature leukocytosis or treatment with sulphonamides or penicillin Other investigators[24, 25] have denied the occurrence of a regular leukocyte rhythm Many factors governing or influencing the release of mature forms have been reported and some of these will probably be considered by other papers in this monograph A few interesting factors might be mentioned The smolar concentration of extracellular fluids which gave a proportional and selective granulocytosis paralleling the increase in molarity of

THE NEUTROPHILIC LEUKOCYTE

By Joseph C Sieracki*

Department of Laboratories Henry Ford Hospital Detroit Mich

The purpose of this communication is to review briefly some of the known properties of the human neutrophilic leukocyte and to correlate these properties with various purported functions where possible In this review not all of the investigated reports are listed More emphasis has been placed on some of the more recent developments especially in regard to leukocytic function Many of the topics will be discussed in detail by more qualified experts in this monograph and accordingly only brief mention of such findings as they relate to the neutrophil of man will be made here This paper in a sense is a restricted review or perhaps more of a running sketch of the biology of this cell

In some of the sources examined it was difficult to ascertain whether the word *leukocyte* was used in its collective fashion or whether it was used specifically for the neutrophilic granulocyte Also in some of the studies no differential count of the white blood corpuscles is given The difficulties in attempting to compare the results of different workers were well expressed by Beck and Valentine [1] who listed three categories as follows

(1) Materials studied varied widely in species of origin anatomical source cell type stage of maturity and mode of preparation

(2) There were different analytical methods and incubation media

(3) Metabolic data have been referred variously to dry weight cell numbers and cell nitrogen

In the same review the authors give some conversion factors which are helpful as reference bases in such studies

The results of this communication will be limited to the human neutrophilic leukocyte for it is well known that structural similarities in the leukocytes of different species may well mask cytochemical disparities A number of these have been found in the literature and they are not only one of degree but may represent complete absence or presence of a given investigated property or function For example it can be found in the work of Wachstein that the neutrophils of man and the heterophil leukocytes of guinea pigs rats and rabbits show marked alkaline phosphatase content while those of the mouse and dog show no evidence of this activity Likewise he found[3] that while 90 per cent of the neutrophils in man were strongly positive for glycogen only faint traces were found in the rat and mouse The alkaline phosphatase[4] was found to be present in eight times the concentration obtained for human cells under the same *conditions when the cells of the rabbit were studied* Barnes found no evidence of trypsin in the heterophil of the rabbit but marked evidence of activity in the cat The opposite was true for nuclease Many such examples could be cited

The history of the human neutrophilic leukocyte is well treated by Rolleston[6] and in many of the current textbooks and need not be considered in detail here

* Present address The Cancer Research Institute New England Deaconess Hospital Boston Massachusetts

parent for four days Some probably die four days after entering the circulation and he suggests 10 days as a conservative average length of life This suggestion is interesting in the light of the studies of Roberts and Kracke [33] who postulated a circulatory or intravascular phase of four days on the basis of a study of a case of agranulocytosis These investigators observed that the neutrophils may be entirely gone from the peripheral blood within four days after their number began to diminish Wright s estimate[16] gave an average of 14 days as the time from the blast form to the mature neutrophilic leukocyte White [34] using transfused leukocytes tagged with atabrine mentioned that these cells do not remain in the peripheral blood for more than one to two hours and do not produce significant changes in the leukocyte count The granulocytes were not separated from the nongranular cells and refer only to the total white cell numbers The author also states that a leukocyte may have many intravascular life spans for the cells move freely from their vascular channels to the tissues and vice versa Kline [35, 36] using radioactive phosphorus found an average life span of 13 days for leukocytes

The *in vitro* survival time also varies considerably Simpson [37] reported that pus cells in the sputum remained viable for nine days at room temperature Osgood[38] found neutrophils surviving 2 to 4 days in tissue culture of human bone marrow Tullis [5] following his method of separation and preservation found that the leukocytes had a variable viability ranging from one to four weeks He also states that leukocytes are disintegrated in the blood at body temperature from three to five days Jeanneret and Fischer[39] described viable neutrophilic leukocytes 70 to 80 hours when they were kept in citrated blood Scudder *et al* [40] showed motile leukocytes in blood stored in ACD solution for 8 to 18 hours

Kelemen[41] said recently According to our present knowledge the life span of circulated leukemic leukocytes hardly exceeds 12 to 48 hours Osgood *et al* [42] measured the uptake of radioactive phosphorus in isolated DNA and calculated a 3-day life span in patients with chronic granulocytic or subacute monocytic leukemia A word of caution as to the validity of Osgood s method[43] as originally reported has been recorded [44] The eventual fate including the sequestration and visceral circulation of leukocytes will be elucidated for us by Doctor Bierman

There are many methods described in the literature for the study of leukocytes Of recent interest are the numerous recent techniques which have been suggested for the separation of white cells [45-51] Many of these techniques utilize various agents which allow gravitational force to aid in a differential separation of the formed elements Other techniques utilize nontoxic hemagglutinins electrophoresis specific antiserum and other cell toxins Some of the problems involved are discussed by Tullis who also describes a method of preservation of the separated white cells [28, 5]

The mature neutrophilic leukocyte has been briefly characterized by Bunting[52] as a differentiated end cell without the power of reproduction characterized by an eccentric horseshoe shaped or S shaped lobed nucleus and by specific fine granulation of its cytoplasm Functionally it is marked by extremely active ameboid motion and by the power of phagocytosis Morpho

these fluids was reported by Tullis[26, 27] The factor of adrenal hormones in this mechanism has been considered by the author[28]

Dietary factors have long been a source of discussion in their effect on the release of cells One of the earlier workers Uden[29] felt that a diet which increased the blood cholesterol decreased the lymphocytes of the blood and caused a significant increase in the granulocytic neutrophils Even more striking, she showed a marked increase of bizarre neutrophils along with the neutrophilia Garrey[30] reported on the nonexistence of digestive leukocytosis Wright[15] encourages dietary restriction in the treatment of leukemias avoiding those factors in granulocytic leukemias which might allow the nucleoproteins to stimulate myelopoiesis further Bone marrow pressure alterations[31] offer a new factor to be considered in release of blood cells from the marrow

Most of the more common causes of neutrophilia are associated with various tissue destructive processes, such as acute infections, chemical and metabolic intoxication neoplasms thromboses postoperative states hemolytic anemias, *etc* Some of the co called physiological factors are strenuous exercise, repeated vomiting tachycardias vitamins hormones pregnancy *etc* A discussion of variations in leukocyte numbers under the influences mentioned is beyond the scope of this paper

The next step in the study of the biology of these cells is the concept of the average length of life and the life span These are different concepts The life span is usually taken to mean the time limit beyond which the viability of cells does not extend even under the *most favorable circumstances* The average length of life of an individual neutrophil probably begins when the last myelocyte divides to produce daughter cells Since it is difficult to define this last point or follow the individual neutrophilic leukocyte the figures for average length of life and the so-called life span ' are many and varied

The various *in vitro* life spans add further difficulties in evaluating the important measurement of the time from cell birth to cell death The average length of life or normal life expectancy could be used to define the average or normal expected time between birth and death of a given cell type under normal conditions the life span as defined above being the time limit beyond which the viability of the cells does not extend even under the *most favorable circumstances* The various *in vitro* measurements of this interval are perhap better termed survival times under specified environmental conditions *v* survival time in tissue culture ACD solutions *etc*

The average length of life is known to consist of a number of stages The maturation time or phase represents the first interval from the birth of a cell in the hematopoietic tissue to its release from this tissue into the blood the intravascular or circulatory phase being from the time the cells enter the blood stream until they leave the vessels The migratory tissue phase would be the time sp nt in the tissues *i e* extravascularly Obviously the cells may have numerous intervals classified as intravascular and migratory phases The final or definitive phase might well be called the sequestration or terminal phase and this phase may occur intravascularly or extravascularly Cowdry[3] mentioned an interesting assumption namely that the individual neutrophils of man live in the bone marrow after their birth with the last mitosis of their myelocytic

with a marked tendency to cytological homogeneity some groups containing only granulocytes while others contain the other cell components of the peripheral blood It is Fleck s contention that only inflammatory factors can provoke leukergy [67] He believes the mechanism of leukergic clumping consists in random collision of the sticky cells and most probably not in their attraction at a distance This theory has not met with uniform acceptance for as Moeschlin states [68] Fleck speaks of leukocytic agglutination in incubated citrated blood as leukergy an expression which appears confusing to us and which we therefore prefer not to use

Some of the physical properties of the mature neutrophils are a specific gravity of 1 065 [8] migration toward the anode [69] cataphoretic velocity slower than that of the lymphocyte [70] They are preferentially wet by water [71] show a fragility which is inversely proportional to the content of vitamin C whose integrity is best maintained in isotonic or slightly hypertonic solutions [25] The electrical surface charge and the surface tension are believed by Fritze to govern the emigration of the granulocytes to an area of inflammation the ameboid movement of the mature granulocytes their phagocytic power and their tendency to aggregate

The water content of human white cells is probably about 80 per cent Some of the available figures from other species show 82 per cent in the horse[72] and 79 59 per cent for the rabbit [73] No data on the normal electrolyte content for human white cells was found Wilson and Mannery[74] reported their findings in the rabbit The concentration in horse leukocytes given by Endres and Herget[72] lists the following per cell

Sodium—113 mM/L
Potassium—23 mM/L
Calcium—2 mM/L
Chlorides—70 mM/L
Bicarbonate ion—1 8 mM/L
Phosphorus—19 mM/L

These authors[72] also comment on the rather marked anion deficit of 47 mM or 34 per cent of the cation concentration This finding is interesting in view of the recent communication by Shenk [75] who showed that the Parr bomb and microcarrius methods for tissue chlorides give much higher values than those obtained with the Van Slyke method as used by Endres and Herget

The recent work by Vallee and his associates[76, 77] on zinc provides a figure for this trace element to be 3 2 + or − 1 3 × 10 to the minus 2 Gamma of zinc per million cells An interesting finding is the marked reduction of zinc in patients afflicted with leukemia to 10 per cent of this amount A rise in the amount following therapeutic amelioration of the leukemic process is as yet unexplained [78] The threefold to tenfold elevation of this zinc content in patients having refractory anemia accompanied by leukopenia has been reported [79] Further work to help elucidate the exact role of zinc is in progress [80]

Using the platelet white blood cell layer Bodansky et al [81] have confirmed the earlier work on the normal white cell ascorbic acid level and its ability to reflect the body store of this vitamin The decreased levels in the white cells

logically there have been many recent interesting observations both on living and fixed neutrophils using many and varied techniques Some of these observations will be cited here briefly Schwind[54] has compared the supravital and air dried smear techniques Block[55] has reported an adaptation of the Maximow technique for the study of sections of hematopoietic tissues Bessis and others[56, 57, 58, 58a] have presented some fascinating work on phase microscopy of these leukocytes

Electron microscopy of human white blood cells has a steadily increasing bibliography[58, 60, 61, 6] since the original observations by Rebuck and Woods[59] made in the laboratory of the Henry Ford Hospital Detroit, Mich These investigators studied air dried and fixed smears and imprints of peripheral blood bone marrow and inflammatory exudate cells These studies of the granules revealed some interesting features The granules are round oval, or rod shaped *vacuolar* structures measuring 70 × 85 mμ to 465 × 650 mμ These apparent vacuolar structures permit electrons to pass through their substance with little electron scattering This finding suggested a content rich in compounds of low molecular weight Close scrutiny of these compounds showed a slight increase in density at the periphery This finding is more pronounced in exudate cells In using unfixed neutrophil granules and submitting these to desiccation by electron bombardment Rebuck[61] showed the decrease in size of a neutrophil granule from 260 × 340 mμ to 220 × 260 mμ This shrinkage believed to be due to aqueous content was less pronounced than in eosinophil granules when subjected to this treatment under comparable conditions The shrunken granules contain a homogenous substance offering appreciable resistance to the passage of electrons

With the development of methods of fixation[63] and handling electron microscopy of sectioned leukocytes has been made possible Kautz and DeMarsh[64] have recently published the first electron micrographs of leukocytes The neutrophils showed a typical nuclear shape with a finely precipitated homogenous matrix and a thin membrane The cytoplasm showed less scattering power than the nucleus Neutrophilic granules were homogenous and somewhat dense ranging $^1/_{10}\ \mu$ to $^1/_4\ \mu$ and generally showed a clearly defined membrane Granular and filamentous mitochondria have been depicted[65]

One peculiar property of the leukocytes and of the neutrophils in particular is their adhesiveness or stickiness *in vitro* Tullis[9] divides this adhesiveness into two categories agglutination and clumping Agglutination is shown only by dead cells which exhibit no ameboid motion no phagocytosis do not breathe and cannot be separated from the group This type of adhesion is seen in all adverse conditions where cell death has occurred

Clumping is temporarily reversible However if this process is allowed to continue for one to two hours death ensues It is commonly seen after exposure to red cell sedimenting agents Even more interesting is the as yet unexplained action of the acetate ion in preventing this clumping The cells after such clumping can be separated and resuspended Once the clumping has occurred however the *in vitro* survival time is shortened

The concept of leukergy has been championed by Fleck who first reported it in 1942[66] In this phenomenon groups of 3 to 20 or more cells are formed

the stage of maturation Jacobson and Webb[99] using cytological and enzymatic methods studied the distribution of the two types of nucleic acids during mitotic division Further work on the nucleic acids in leukemic cells[100] and pernicious anemia[101] has been presented recently White *et al*[102] studied DRNP in purulent sputum while Menten[103] did chemical analyses of neutrophils in abscesses of patients DNA phosphorus and RNA phosphorus of degenerating cells showed a statistical relationship to the bacteria which gave rise to the infection

The lipid content of leukocytes has a long and interesting history Boyd[104] did a chemical analysis on the white cell fraction of blood of young women and found 1 7 per cent lipoids Of this small amount 47 per cent was phospholipid while 16 per cent represented free and combined cholesterol A total fatty acid analysis on the leukocyte fraction showed it was 65 per cent of total lipoid The histochemical approach using Sudan dyes has intrigued a number of investigators Of more recent interest has been the use of Sudan Black B[98, 105, 109] which did not stain the nucleus but demonstrated or produced granules in the cytoplasm of myeloid cells which were shown to increase in number with increasing cell maturity The concept of stable sudanophilia has recently been well reviewed[110] and discussed as a chemical combination of the dyes with cytoplasmic constituents rather than on lipoid staining Bloom and Wislocki[111] using Baker s acid hematein test and pyridine extraction have shown negative reactions of nuclei of myeloid cells positive staining of mitochondria and myeloid granules Whether all these elements which stain positively actually contain phospholipid is not certain

β glucuronidase activity has been demonstrated in human white cells It is slightly greater in the neutrophil than lymphocyte (5 4)[112] There have been recent attempts[113, 114] to study variations of this activity in various diseases The glucuronic content of leukocytes was reported recently[115] and it may have some relation to the enzyme activity

The phosphatases have been studied extensively in the past decade Wachstein[3] Deane[116] Rheingold and Wislocki[96] Rabinovitch and Andreucci[117] and Storti *et al*[118] have reported their histochemical findings on myeloid cells The nuclei are generally positive with alkaline phosphatase techniques while the acid active enzyme shows a variable nuclear reaction The metamyelocytes and segmented neutrophils show positive cytoplasmic reactions with each Younger cells give a negative cytoplasmic reaction for alkaline phosphatase while there is a positive reaction of myeloid granules from blast to mature forms Cram and Rossiter[119] showed 1000 more times of alkaline phosphatase activity in granulocytes than in serum Using Rossiter s technique Valentine[120] has investigated the quantitative variations in various maladies Nucleotidase activity which is 10 times greater in granulocytes as compared with lymphocytes has been studied in leukemic patients[121]

Diastatic activity when present[122] has been reported as highly suggestive but not absolutely conclusive[123] in human neutrophils Wagner[124] believes that degradation of glycogen in human neutrophils is not the result of diastatic enzyme activity

Proteolytic enzyme activity of neutrophils includes a battery of various

of patients having cancer and other chronic diseases is associated with an excessive catabolism of protein Folinic acid content (as opposed to free folic acid and folic acid conjugase) was greater in leukemic leukocytes as compared to normals in the study of Swendseid *et al* [82] The increase was correlated with the degree of cell immaturity

Thiamin values are three times normal in leukocytes of patients having leukemia or Hodgkin s Disease or carcinoma of gastrointestinal tract but are decreased in those having portal cirrhosis of the liver [83]

There are many reports in the literature on various chemical constituents and staining reactions of leukocytes The limitations expressed by Beck and Valentine[1] on attempting to evaluate these constituents and reactions have been mentioned earlier The evidence presented comes from varied techniques many of which have not been completely evaluated A thorough review of them is beyond the scope of this paper Furthermore Doctors Martin Valentine Fleischer, Seabra and Wachstein discuss chemical and metabolic aspects of leukocytic activity in Part V of this monograph The brief discussion that follows will accordingly be limited to a survey of the chemical constituents and staining reactions of the human neutrophilic leukocyte

Glycogen content of neutrophils has been studied via histochemical and microchemical methods [84-91] While progranulocytes and all nuclei have no demonstrable glycogen the other more mature myeloid cells show an extragranular distribution Neutrophils from exudates show a more diffuse distribution The normal values for mature neutrophilic leukocytes has been calculated to be 4 23 micrograms per million cells [89] Elevated values are reported in glycogen storage disease [89] granulocytic leukemia [90] and polycythemia vera [87] The glycogen content of normal white blood cells (wet cells 0 17–0 67 per cent) is within the same range as that of striated muscle [91] No significant amounts are demonstrable in the nongranular leukocytes

The nucleic acids and nucleoproteins in neutrophils has likewise been the subject of much investigation LaCour [92] using the Pappenheim and Feulgen stains did some beautiful work on cell differentiation in the blood It was his contention that differences in concentration of nucleic acids may be responsible for the proportion of red and white cells in the blood His explanation for the failure of the heterochromatin of stab leukocytes to be stained frequently and variably was a decrease in the concentration of nucleic acids in the parent cells White[93] reported on the possible relation of basophilia of cells as a reflection of nucleic acids Van den Berghe[94] believed RNA may be a factor responsible for the affinity of Giemsa staining Thorell s[95] monograph described his studies showing a general tendency for a fall in ribonucleoprotein with increased cell maturity He used both cytochemical stains and quantitative microspectrophotometric techniques Rheingold and Wislocki[96] showed that all nucleoli of granulocytic series contained RNA but only the cytoplasm of myeloblasts and progranulocytes were diffusely positive with the Feulgen stain Similar studies were reported by Gardikas and Israels [97] The work of Osgood[4] [43] and Kline[35] [86] using the uptake of radioactive phosphorus in DNA has been mentioned Davidson *et al* [98] showed that the amount of DNAP cell was relatively constant in health and leukemia while RNAP/cell varied with

viability and suggests that a leukocyte loses physiological functions at variable times in a descending order: oxidative metabolism, phagocytosis, motility, resistance to impermeable dyes, Brownian movement, and morphologic integrity. Kerby[144, 145] has presented a method for the detection of leukocyte injury based on the release of a lysozyme-like enzyme.

The ameboid motion of leukocytes has been the subject of much study and this topic will be considered in detail elsewhere in this monograph. The neutrophilic leukocytes move at a rate of 30.8 μ to 53.9 μ per minute.[146] This rate is variable depending on methods of study, environment, species, *etc.*

Lewis[147] studied the motion of rat granulocytes and offered an interpretation of the manner in which this motion is accomplished. De Bruyn[148, 149] has interpreted the ameboid motion of rabbit white cells in the light of Frey-Wyssling's structural concept for protoplasm. Mallery and McCutcheon[150] have shown that neutrophils of acutely ill patients moved more slowly than healthy controls. One of the first investigators to show that ameboid motion does not run parallel to phagocytosis was de Haan.[151] Recognition of this fact was recently confirmed by Matoth[15] who showed a marked difference, in the number and quality of ameboid neutrophils in Hank's medium, between newborn and adult cells. The newborn cells were sluggish, almost round with plump pseudopods. When adult serum was added they were indistinguishable from adult cells in motility, but showed a marked difference in phagocytic ability.

Phagocytosis is a well known function of the neutrophil and is well documented in the literature.[153-157] Of recent interest is the work of Matoth[152] showing considerably less phagocytosis by neonatal neutrophils as compared with adult cells *in vitro*. Jersild's[158] experiments showed that no cell younger than metamyelocytes was capable of phagocytosis and in acute blastic leukemias Jersild was able to show many peroxidase positive cells which showed no phagocytic ability. In general, human granulocytes show decreased phagocytic ability with decreased maturity. Lerner[159] has recently reopened the question of "surface phagocytosis" while Tullis[9] reports on a phagocytosis promoting factor. The adrenal cortical activity appears to have a variable effect on neutrophilic phagocytosis often depending on degree of such activity. In humans, Rebuck *et al.*[160-163] and others[164] have shown inhibition of phagocytosis while Moeschlin[165] reports no effect on neutrophils.

The importance of phagocytosis in defense functions is basic and needs no lengthy discussion. Ehrlich[166] relates that the role of the neutrophilic granulocyte is the destruction of antigen in the essential phase of inflammation. Though Rogers and Tompsett[167] believed they demonstrated the survival of pathogenic staphylococci in human neutrophils, the validity of the technique has been questioned by Braude and Feltes.[168]

Chemotaxis is especially well demonstrated by the neutrophil and can be exhibited in attraction or repulsion for a distance of about 1 mm. Doctor McCutcheon, who is eminently more qualified to discuss this interesting biologic phenomenon, has written about the subject in 1946[169] and in 1952.[140] Since this last report, there have been a few interesting communications on this subject. Harris,[170] using a new technique, believes that altered tissue and exudates

activities under a number of names [1 3] Leukoprotease with marked fibrinolytic activity was reported by Opie [1 5, 1 6, 127] Tryptic,[78, 1??, 123] peptidase [9] peptic [28] and catheptic [-8, 1??] activities have been reported

Lipase activity has been demonstrated in the neutrophil,[9 1] and it has been suggested that it may well be a common esterase [9] Rossiter and Wong[1 8] have shown this to be true for the rabbit while also showing the absence of cholinesterase in white cells The esterase in the rabbit white cells hydrolyzes triglycerides of low molecular weight fatty acids

The histamine content of white cells has been well reviewed recently by Code [-8] Though evidence pointed to myelocytic cells as the histamine cells the neutrophil of humans does not carry histamine A more recent paper implicates the basophil [1 9]

Agner isolated a ferment from myeloid leukocytes of humans which he termed verdoperoxidase (V P O) [130] This enzyme was recovered from empyema fluid white cells of a patient with granulocytic leukemia and chloroleukemic infiltrates V P O is found only in significant amounts in myeloid cells and constitutes from 1 to 2 per cent of dry weight of leukocytes [131] Humble[1 2] believed the green pigment of chloroma to be related to choleglobin The histochemical aspects of this enzyme and its method of demonstration have been challenged recently as have the other oxidative reactions of granulocytic cells [133, 134]

Many enzymes of carbohydrate metabolism of leukocytes have been investigated Much of the recent work has been well reviewed by Beck and Valentine [1] McKinney Martin *et al* [135] present evidence of a Krebs cycle activity in mature neutrophils Wagner[136] reports that the glycolysis of horse dog and human are approximately the same and he shows that leukocyte glycolysis occurs in lymphocyte suspensions and is not specific for neutrophilic leukocyte Glyoxalase[137] activity has also been demonstrated Traces of catalase have been mentioned in the literature [9] Laves and Thoma [138] in their investigations of granules of white cells report that the neutrophilic granule probably contains hyaluronic acid ester Seabra[139] has demonstrated the acid alcohol fastness of these granules Allen[140] mentions that human granulocytes are dopa (tyrosinase) positive Lajtha *et al* [141] showed the uptake of S^{35} sulphate by myeloid cells only in cultures of bone marrow which is cytoplasmic and is not associated with protein They believe it represents an unknown specific function Wachstein[142] showed more reducing activity of exudate and more mature granulocytes but no difference between leukemic and normal cells of the same type Weiss[143] *has investigated the binding of acid and basic dyes* at various pH ranges

In determining some of the functions of leukocytes especially the mature granulocytes the state of viability must be ascertained One of the more simple techniques was reported by Simpson [87] who used a dilute solution of methylene blue Simpson s observations extended over a six year period of study of pus cells in sputum Wilson and Mannery[74] suggested that the impermeability of the rabbit s white cells to trypan blue was a useful criterion In their hands the test correlated with motility studies and with chemical evidence of the maintenance of concentration gradients between intracellular and extracellular fluids Tullis [8] considers the phagocytic index a standard test for *in vitro*

viability and suggests that a leukocyte loses physiological functions at variable times in a descending order oxidative metabolism phagocytosis motility resistance to impermeable dyes Brownian movement and morphologic integrity Kerby[144,145] has presented a method for the detection of leukocyte injury based on the release of a lysozyme like enzyme

The ameboid motion of leukocytes has been the subject of much study and this topic will be considered in detail elsewhere in this monograph The neutrophilic leukocytes move at a rate of 30 8 μ to 53 9 μ per minute[146] This rate is variable depending on methods of study, environment species *etc*

Lewis[147] studied the motion of rat granulocytes and offered an interpretation of the manner in which this motion is accomplished De Bruyn[148,149] has interpreted the ameboid motion of rabbit white cells in the light of Frey Wyssling s structural concept for protoplasm Mallery and McCutcheon[150] have shown that neutrophils of acutely ill patients moved more slowly than healthy controls One of the first investigators to show that ameboid motion does not run parallel to phagocytosis was de Haan[151] Recognition of this fact was recently confirmed by Matoth[152] who showed a marked difference in the number and quality of ameboid neutrophils in Hank s medium between newborn and adult cells The newborn cells were sluggish almost round with plump pseudopods When adult serum was added, they were indistinguishable from adult cells in motility but showed a marked difference in phagocytic ability

Phagocytosis is a well known function of the neutrophil and is well documented in the literature[153-157] Of recent interest is the work of Matoth[152] showing considerably less phagocytosis by neonatal neutrophils as compared with adult cells *in vitro* Jersild s[158] experiments showed that *no* cell younger than metamyelocytes was capable of phagocytosis and in acute blastic leukemias Jersild was able to show many peroxidase positive cells which showed no phagocytic ability In general human granulocytes show decreased phagocytic ability with decreased maturity Lerner[159] has recently reopened the question of surface phagocytosis while Tullis[160] reports on a phagocytosis promoting factor The adrenal cortical activity appears to have a variable effect on neutrophilic phagocytosis often depending on degree of such activity In humans Rebuck *et al*[162,163] and others[164] have shown inhibition of phagocytosis while Moeschlin[165] reports no effect on neutrophils

The importance of phagocytosis in defense functions is basic and needs no lengthy discussion Ehrlich[166] relates that the role of the neutrophilic granulocyte is the destruction of antigen in the essential phase of inflammation Though Rogers and Tompsett[167] believed they demonstrated the survival of pathogenic staphylococci in human neutrophils the validity of the technique has been questioned by Braude and Feltes[168]

Chemotaxis is especially well demonstrated by the neutrophil and can be exhibited in attraction or repulsion for a distance of about 1 mm Doctor McCutcheon who is eminently more qualified to discuss this interesting biologic phenomenon has written about the subject in 1946[169] and in 1952[160] Since this last report there have been a few interesting communications on this subject Harris[170] using a new technique believes that altered tissue and exudates

(dead granulocytes, crushed and autolyzed tissues, enzymatic digests) were not chemotactic for human neutrophils He also mentioned that if hyperlipemic or freshly drawn serum is used, the leukocytes had a sluggish movement Kuna and Chambers [171] working with rat granulocytes suggest the possibility that glycogen is the natural granulocyte attracting agent released on injury of tissue Moon *et al* [17?] working with rabbit cells, suggests that normal tissues contain chemotactic substances which are released from areas of injury and initiate the complex activities of acute inflammation Leukotaxine and exudin have been discussed by Menkin[173, 174] recently in relation to ACTH (corticotropin) and cortisone in inflamed areas Menkin s interesting experiments were conducted on rabbits and dogs

Adherence to endothelium, subsequent diapedisis and eventual emigration to areas of inflammation even in relatively avascular tissues are other well known biologic properties or functions of leukocytes Rebuck has shown that neutrophils are seen as soon as 30 minutes after an inflammatory stimulus is instituted in his human skin windows [175] The cellular exudate consists of neutrophils and histiocytes for the first 8 hours After performing their phagocytic functions they lose much of their granule containing cytoplasm in the form of small fragments [176] These free particles are then ingested by macrophages and may represent a trephocytic type of function as described by Carrel [177] This particulate transfer of cytoplasm is believed to account for the rapid assumption of peroxidase positive cytoplasm[178, 179] and enhanced Nadi reaction[180] by lymphocytes and macrophages in areas of acute inflammation Such a transfer could work in the opposite direction (*i e* from lymphocyte to neutrophil) and its importance in biochemistry and histochemistry of leukocytes bears further investigation

The proteolytic enzymes find their usefulness in the digestion of bacteria and other inflammatory products [17] *e g* fibrin network of pneumonia and necrotic *tissues of furuncle* [1 6] *In this regard it was Crob*[181] *who found that the products* of hydrolysis of leukoprotease or trypsin in the cat and rat could stimulate bacterial growth and could inhibit sulfonamide action directly and indirectly

Cram and Rossiter[119] found 1000 times more alkaline phosphatase activity in the granulocytes than in serum and suggested that they are the major source of serum alkaline phosphatase Wachstein and Fell[18?] have demonstrated evidence of increased enzyme activity in neutrophils in inflammatory processes Storti *et al* [183] have recently summarized the literature on various techniques in determining phosphatase activity They relate their results to the distribution of the lipids polysaccharides and nucleic acids and to the enzyme in the same cell The metabolic parameters or biochemical signatures of various disease processes involving leukocytes has been discussed by Valentine[1, 184] and by others [114] *e g* in leukocytosis the alkaline phosphatase activity may be five times normal values while in chronic myelogenous leukemia the value is significantly lower

The role of β glucuronidase has not been definitely resolved Rossiter and Wong[11] speculated that it may be part of the complex tissue reaction of inflammation via hydrolysis of glucuronic acid derivatives Follette *et al* [114] sug

gest a possible relationship between glucuronic acid and the enzyme in cellular detoxification Meyer *et al*[185] do not believe that monosaccharides can be produced from hyaluronate when previously exposed to hyaluronidase in the human body This production occurs *in vitro* as they have shown However it has not been proved *not* to occur under the complex reactions in inflammatory tissues which may have in their environment many ancillary factors The fate of aldobionic acid derivatives isolated from various types of pneumococci has not been thoroughly investigated

Bokretas[186] in 1937 showed that the cholesterol of leukocytes decreased with pyrexia in infective diseases and increased after fever He found free cholesterol in the cells and believed it was concerned with the fixation of toxins and the formation of immune bodies Any confirmation of this work has eluded me

Verdoperoxidase constitutes 1 to 2 per cent granulocytes according to Agner who suggested that because of its peroxidatic activity uric acid is oxidized to an intermediate that inactivates diphtheria toxin and because of the similarity of its absorption spectra to cytochrome A it may be a respiratory pigment[130, 187, 188] The latter function has been seriously questioned by Chance[25] on the basis of his spectrophotometric studies Delaunay[189] on the basis of enzyme inhibitors in his work on chemotaxis believes that the abundance of peroxidase in neutrophilic leukocytes as compared with lymphocytes is related to their migratory power Two recent reports are interesting on this extraordinary component Schultz *et al*[190] in studying chloromatous tumors of rats produced by injection of material from a patient with granulocytic leukemia find verdoperoxidase in concentrations up to five times higher than that reported by Agner The other report is one by Maehly and Chance[124] recommending guaiacol and pyrogallol as histochemical staining reagents for peroxidase These investigators also state it is much more difficult to avoid interference from oxidase action however in whole cells than in solution No reliable methods are known to the authors that would allow a sharp discrimination between oxidase and peroxidase activities *in vivo* The use of carbon monoxide or potassium cyanide as test inhibitors has been proposed Bennett[191] reported the presence of a fever producing factor extracted from animal tissue which differed from Menkin s pyrexin

Bunting[54] mentions the possibility that like other tissue cells the neutrophilic leukocyte may yield thromboplastin and hence aid in the coagulation of blood Leukocytic secretory function which includes that of the granulocytes is discussed in this monograph by Doctor Richter Duran Jorda[192] contends that the neutrophil secretes pus and in the process changes its size and nuclear structure and becomes a mononuclear Eventually he believes it becomes a lymphocyte

In 1939 Williams and Dougherty[193] reported on the increase in complement activity of guinea pig serum following injection of aleuronat Since this activity produced a heterophilic leukocytosis the possibility of heterophils as a source of serum complement was raised

The existence of A B O blood group antigens in leukocytes has been reported briefly by Twible[23] There was no distinction made between the granular and

nongranular white cells Immunologic investigations and reports on leukocytes are appearing more frequently in the recent literature[8 63 194 19] and, though interesting they do not merit a discussion by themselves

There are many facets of the biology of the neutrophilic leukocyte which have not been considered adequately in this running sketch Some of these facets are considered later in this monograph The human neutrophilic leukocyte has been a stimulus for many reports in the literature As yet knowledge of specific mechanisms and functions of these leukocytes is at a primitive level The facts are few the unanswered questions are many There is little doubt that this monograph will contribute to a better understanding of some of these problems

The author wishes to express his sincere gratitude to his wife and to the many members of the Henry Ford Hospital staff who have helped in the preparation of this manuscript

Bibliography

1 Beck W S & W N Valentine 1953 Cancer Research 13 309
2 Wachstein M 1946 J Lab Clin Med 31 1
3 Wachstein M 1949 Proc Soc Exptl Biol Med 72 175
4 Haight W F & R J Rossiter 1950 Blood 5 267
5 Barnes J M 1940 Brit J Exptl Path 21 264
6 Rolleston H 1934 Proc Roy Soc Med 27 1161
7 Naegeli O 1931 Blutkrankheiten und Blutdiagnostik 5th ed Springer Berlin Germany
8 Minot C S 1912 Human Embryology 2 517 E Keibel & F P Mall Eds Lippincott Philadelphia Pa
9 Albritton E C (Ed) 1952 Standard Values in Blood 67–69 Saunders Philadelphia Pa
10 Wintrobe M M 1951 Clinical Hematology 43 Lea & Febiger Philadelphia Pa
11 Krumbhaar E B 1932 Am J Med Sci 184 215
12 Jacobson L O E K Marks M J Robson E Gaston & R E Zirkle 1949 J Lab Clin Med 34 1358
13 Jacobson L O E L Simmons E K Marks E O Gaston M J Robson & J H Eldredge 1951 J Lab Clin Med 37 683
14 Lorenz E C Congdon & D Uphoff 1951 Radiology 58 863
15 Wright C S 1950 Am J Roentgenol Radium Therapy 64 907
16 Wright C S 1953 Blood Cells and Proteins 284 Academic Press New York N Y
17 Doan C A L G Zerfas S Warren & O Ames 1928 J Exptl Med 47 403
18 Sabin F R R S Cunningham C A Doan & J A Kindwall 1925 Bull Johns Hopkins Hosp 37 14
19 Sabin F R 1928 Physiol Revs 8 91
20 Smith C & A M McDowell 1929 Arch Internal Med 43 68
21 Garrey W E & V Butler 1929 Am J Physiol 2 90
22 Garrey W E & V Butler 1929 Proc Staff Meetings Mayo Clinic 4 20
23 Koch E & P Lubbers 1949 Deut Arch klin Med 196 268
24 Casey A E 1940 Proc Soc Exptl Biol Med 45 863
25 Kennon B R M E Shipp & D C Hetherington 1937 Am J Physiol 118 690
26 Tullis J L 1947 J Clin Invest 26 1098
27 Tullis J L 1948 Am J Med Sci 215 424
28 Tullis J L 1953 Blood Cells and Plasma Proteins 265 Academic Press New York N Y
29 Luden G 1917 J Lab Clin Med 3 141
30 Garrey W E & W R Bryan 1935 Physiol Revs 15 597
31 Petrakis N L 1954 J Clin Invest 33 27
32 Cowdry E V 1942 Problems of Ageing 679 Williams & Wilkins Baltimore Md

33 Roberts S I & K K Kracke 1930 J Am Med Assoc 95 80
34 White L P 1954 Blood 9 3
35 Kline D L & F I Clifton 19 2 Science 115 9
36 Kline D L & I I Clifton 19 2 J Applied Physiol 5 9
37 Simpson H K L 1943 Brit J Exptl Path 24 218
38 Osgood E E 193 J Am Med Assoc 109 933
39 Jeanneret H & K Fischer 1941 Schweiz med Wochschr 71 204
40 Sculler J C K Drew I Tuthill & M F Smith 1941 Bull N Y Acad Med 17 2 3
41 Kelemen F C Bikich J Barbola & B Tana 1953 Acta Hemat l 9 171
42 Osgood E E H Tivey K B Davison A J Seaman & J C Li 1952 Cancer 5 331
43 Osgood E E J C Li H Tivey M L Duerst & A J Seaman 1951 Science 114 9
44 Cunningham L 1951 Science 115 504
45 Vallee B L W L Hughes Jr & J C Gibson 1947 Blood Special Issue 1 82
46 Spear F 1948 Blood 3 10 5
47 Minor A H & L Burnett 1949 Blood 4 6
48 Li J C & E Osgood 1949 Blood 4 6 0
49 Buckley E S M J Powell & J C Gibson 1950 J Lab Clin Med 36 29
50 Riga D A J C Li & E E Osgood 1951 Am J Med 10 7 6
51 Tulli J I 19 2 Blood 7 891
52 Tulli J I 1951 Blood 6 2
53 Bunting C H 1938 Handbook of Hematology Vol 1 Hoeber New York N Y
54 Schwind J L 1950 Blood 5 59
55 Block M et al 1953 J Lab Clin Med 42 145
56 Bessis M & M Bricka 1949 Rev Hematol 4 350
57 Dicombe C 19 0 Acta Hematol 3 151
58 Marchant J 19 2 Quart J Microscop Sci 93 395
58a Felicard A & A Collet 1953 Rev Hematol 8 132
59 Rebuck J W & H Woods 1948 Blood 3 1 5
60 Bessis M 19 0 Blood 6 1083
61 Rebuck J W 1950 Papers of Mich Acad Sci Arts & Letters 36 317
62 Rebuck J W & H I Woods 1949 Federation Proc 8 365
63 Haguenau F & W Bernhard 1952 Exptl Cell Research 3 629
64 Kautz J & Q B DeMarsh 1954 Blood 9 24
65 Kautz J Personal communication
66 Fleck L & Z Murczynska 1942 Medycyna Weterynaryjna No 2
67 Fleck L 1951 Texas Repts Biol Med 9 697
68 Moeschlin S & F Schmid 1954 Acta Hematol 11 241
69 Eliasoph B 1924 Z ges exptl Med 41 317
70 Abramson H A 1924 Am J Med Sci 167 02
71 Mudd S & E B H Mudd 1926 J Exptl Med 43 12
72 Fritze F 1950 Acta Hematol 4 351 also Z J klin Med 147 568
73 Endres C & L Herget 1929 Z J Biol 88 451
74 Wilson D L & J J Mannery 1949 J Cellular Comp Physiol 34 493
75 Shenk W D 1954 Arch Biochem & Biophys 49 138
76 Vallee B L & J C Gibson 2nd 1948 J Biol Chem 176 445
77 Vallee B L & M D Altschule 1949 Blood 4 398
78 Gibson J C 2nd B L Vallee L C Fluharty & J E Nelson 1950 Acta Unio Intern contra Cancrum 6 112
79 Vallee B L & J G Gibson 2nd 1949 Blood 4 455
80 Hoch F L & B L Vallee 19 2 J Biol Chem 195 531
81 Bodansky O F Wroblewski & B Markardt 1952 Cancer 5 678
82 Swendseid M E L H Bethell & O D Bird 1951 Cancer Research 11 864
83 Abel J C A T Cobham L Craver & C P Rhoads 1942 J Clin Invest 21 1 7
84 Mancini R E 1948 Anat Record 101 149
85 Wachstein M 1949 Blood 4 54
86 Wislocki G B J J Rheingold & L W Dempsey 1949 Blood 4 562
87 Gibb R I & R I Stowell 1949 Blood 4 569
88 Eranko O 1950 Ann Med Exptl et Biol Fenn 28 1
89 Wagner K 1947 Am J Diseases Children 73 559

nongranular white cells Immunologic investigations and reports on leukocytes are appearing more frequently in the recent literature[28,65,191,195] and though interesting they do not merit a discussion by themselves

There are many facets of the biology of the neutrophilic leukocyte which have not been considered adequately in this running sketch Some of these facets are considered later in this monograph The human neutrophilic leukocyte has been a stimulus for many reports in the literature As yet knowledge of specific mechanisms and functions of these leukocytes is at a primitive level The facts are few the unanswered questions are many There is little doubt that this monograph will contribute to a better understanding of some of these problems

The author wishes to express his sincere gratitude to his wife and to the many members of the Henry Ford Hospital staff who have helped in the preparation of this manuscript

Bibliography

1 Beck W S & W N Valentine 1953 Cancer Research **13** 309
2 Wachstein M 1946 J Lab Clin Med **31** 1
3 Wachstein M 1949 Proc Soc Exptl Biol Med **72** 175
4 Haight W F & R J Rossiter 19 0 Blood **5** 26[illegible]
5 Barnes J M 1940 Brit J Exptl Path **21** 264
6 Rolleston H 1934 Proc Roy Soc Med **27** 1161
7 Naegeli O 1931 Blutkrankheiten und Blutdiagnostik 5th ed Springer Berlin Germany
8 Minot C S 1912 Human Embryology **2** 517 E Keibel & F P Mall Eds Lippincott Philadelphia Pa
9 Albritton E C (Ed) 1952 Standard Values in Blood 67–69 Saunders Philadelphia Pa
10 Wintrobe M M 1951 Clinical Hematology 43 Lea & Febiger Philadelphia Pa
11 Krumbhaar E B 193[illegible] Am J Med Sci **184** 215
12 Jacobson L O E K Marks M J Robson E Gaston & R E Zirkle 1949 J Lab Clin Med **34** 1358
13 Jacobson L O E L Simmons E K Marks E O Gaston M J Robson & J H Eldredge 1951 J Lab Clin Med **37** 683
14 Lorenz E C Congdon & D Uphoff 1951 Radiology **58** 863
15 Wright C S 1950 Am J Roentgenol Radium Therapy **64** 90
16 Wright C S 1953 Blood Cells and Proteins 284 Academic Press New York N Y
17 Doan C A L G Zerfas S Warren & O Ames 1928 J Exptl Med **47** 403
18 Sabin F R R S Cunningham C A Doan & J A Kindwall 1925 Bull Johns Hopkins Hosp **37** 14
19 Sabin F R 1928 Physiol Revs **8** 91
20 Smith C & A M McDowell 1929 Arch Internal Med **43** 68
21 Garrey W E & V Butler 1929 Am J Physiol **2** 90
22 Garrey W E & V Butler 1929 Proc Staff Meetings Mayo Clinic **4** 20
23 Koch I & P Lubbers 1949 Deut Arch klin Med **196** 268
24 Casey A E 1940 Proc Soc Exptl Biol Med **45** 863
25 Kennon B R M E Shipp & D C Hetherington 193 Am J Physiol **118** 690
26 Tullis J L 194[illegible] J Clin Invest **26** 1098
27 Tullis J L 1948 Am J Med Sci **215** 4[illegible]4
28 Tullis J L 1953 Blood Cells and Plasma Proteins 265 Academic Press New York N Y
29 Luden G 1917 J Lab Clin Med **3** 141
30 Garrey W E & W K Bryan 1935 Physiol Revs **15** 597
31 Petrakis N L 1954 J Clin Invest **33** 2[illegible]
32 Cowdry E V 194[illegible] Problems of Ageing 629 Williams & Wilkins Baltimore Md

148 DeBruyn I 1944 Anat Record 89 43
149 DeBruyn I 1946 Anat Record 95 177
150 Mallery O T & M McCutcheon 1940 Am J Med Sci 200 394
151 deHaan J 1922 Arch ges Physiol Pflügers 194 448
152 Matoth Y 1952 Pediatrics 9 748
153 Mudd S M McCutcheon & B Lucke 1934 Physiol Revs 14 210
154 Strumia M & F Boerner 1937 Am J Path 13 335
155 Hertzog A J 1938 Am J Path 14 595
156 Iams A M & H M Keith 1947 J Pediat 30 123
157 Berry L J & T D Spies 1949 Medicine 28 239
158 Jersild M 1948 Acta Med Scand Suppl 213 238
159 Lerner E M & J Victor 1952 Proc Soc Exptl Biol Med
160 Rebuck J W R W Smith & R R Margulis 1951 Gastroenterology 19 644
161 Rebuck J W R W Smith & R R Margulis 1951 Federation Proc 10 369
162 Rebuck J W R W Smith & R R Margulis 1951 Proc 2nd ACTH Clinical Conf 460–467 Blakiston Philadelphia Pa
163 Rebuck J W & R C Mellinger 1953 Ann N Y Acad Sci 56 175
164 Crepea S B G E Magnin & C A Seastone 1951 Proc Soc Exptl Biol Med 77 704
165 Moeschlin S W Zurukzoglu & J Crabbie 1953 Acta Hematol 9 278
166 Ehrich W E 1949 J Mt Sinai Hosp N Y 15 337
167 Rogers D E & R Tompsett 1952 J Exptl Med 95 209
168 Braude A I & J Feltes 1953 J Lab Clin Med 42 289
169 McCutcheon M 1946 Physiol Revs 26 319
170 Harris H 1953 J Path Bact 46 135
171 Kuna A & R Chambers 1953 J Clin Invest 32 436
172 Moon V H C G Grand & G A Terschakovec 1954 Arch Path 57 44
173 Menkin V 1953 Brit J Exptl Path 34 412
174 Menkin V 1953 Brit J Exptl Path 34 420
175 Rebuck J W 1947 Ph D Thesis Univ of Minnesota Minneapolis Minn
176 Rebuck J W 1949 Anat Record 103 497
177 Carrel A 1924 J Am Med Assoc 28 255
178 Rebuck J W & F A Monaghan 1948 Federation Proc 7 277
179 Rebuck J W 1950 J Lab Clin Med 36 1009
180 Rebuck J W 1952 Federation Proc 2 424
181 Grob D 1950 J Gen Physiol 33 103
182 Tell H B & J F Danielli 1943 Brit J Exptl Path 24 196
183 Storti E S Perugini & A Rossi 1953 Medicina (Parma) 3 333
184 Valentine W N 1951 Blood 6 845
185 Meyer K A Linker & M M Rappaport 1951 J Biol Chem 192 275
186 Bokretas A 1937 Folia Hematol 58 230
187 Agner K 1947 Nature 159 271
188 Agner K 1949 Federation Proc 8 178
189 Delaunay A J Lebrun & M Barber 1951 Nature 16 774
190 Schultz J H Shay & M Gruenstein 1954 Cancer Research 14 157
191 Bennett I L & P B Beeson 1953 J Exptl Med 98 493
192 Duran Jorda F 1945 Nature 156 110
193 Williams O O & T Dougherty 1939 J Immunol 36 559
194 Finch S C J F Ross & F G Ebaugh 1953 J Lab Clin Invest 42 555
195 Dausset J 1953 Presse Méd 61 1533

90 SUTER E 1947 Helv Physiol et Pharmacol Acta 5 6
91 WAGNER R 1947 Blood 2 235
92 LACOUR L F 1944 Proc Roy Soc Edinburgh 62 73
93 WHITE J C 1947 J Path Bact 59 223
94 VAN DEN BERGHE L & J HOFFMAN 1945 Compt rend soc biol 139 374
95 THORELL B 1947 Studies on the Formation of Cellular Substances During Blood Cell Production Kimpton London England
96 RHEINGOLD J J & G B WISLOCKI 1948 Blood 3 641
97 GARDIKAS C & M C G ISRAELS 1948 J Clin Path 1 226
98 DAVIDSON J N I LESLIE & J C WHITE 1951 J Path Bact 63 471
99 JACOBSON W & M WEBB 1951 J Physiol 112 2P
100 MENTEN M L & M WILMS 1953 Cancer Research 13 733
101 WHITE J C I LESLIE & J N DAVIDSON 1953 J Path Bact 65 291
102 WHITE J C P C ELMES & A WALSH 1954 J Path Bact 66 105
103 MENTEN M L & M WILMS 1954 Arch Path 57 147
104 BOYD E M 1933 J Biol Chem 101 623
105 SHEEHAN H L 1939 J Path Bact 49 580
106 MCMANUS J F A 1945 Nature 156 173
107 BAILIFF R N & C KIMBROUGH 1947 J Lab Clin Med 32 155
108 STORTI E & S PERUGINI 1951 Acta Hematol 5 321
109 HAYHOE F G H 1953 J Path Bact 65 413
110 LILLIE R D & H J BURTNER 1953 J Histochem Cytochem 1 8
111 BLOOM M L & G B WISLOCKI 1950 Blood 5 79
112 ROSSITER R J & E WONG 1950 Blood 5 864
113 FOLLETTE J H W N VALENTINE & J S LAWRENCE 1952 J Lab Clin Med 40 825
114 ANLYAN A J 1954 Cancer 7 391
115 FOLLETTE J H W N VALENTINE E B HARDIN & J S LAWRENCE 1954 J Lab Clin Med 43 134
116 DEANE H W 1947 Am J Anat 80 321
117 RABINOVITCH M & D ANDREUCCI 1949 Blood 4 580
118 STORTI E S PERUGINI & V ROSSI 1953 Medicina (Parma) 3 333
119 CRAM D M & R J ROSSITER 1949 Can J Research 27 290
120 VALENTINE W N 1951 J Lab Clin Med 38 39
121 SWENDSIED M E P WRIGHT & F H BETHELL 1952 J Lab Clin Med 40 515
122 MORRIS R S & T R BOGGS 1911 Arch Internal Med 8 806
123 FIESSINGER N 1923 Les Ferments des Leucocytes en Physiologie et Therapeutics Générales Masson Paris France
124 WARNER K 1950 Arch Biochem Biophys 26 123
125 OPIE E L 1906 J Exptl Med 8 40
126 OPIE E L 1910 Arch Internal Med 5 541
127 OPIE E L 1922 Physiol Revs 2 552
128 ROSSITER R J & E WONG 1949 J Biol Chem 180 933
129 CODE C F R G MITCHELL & J C KENNEDY 1954 Proc Staff Meetings Mayo Clinic 29 200
130 AGNER K 1941 Acta Physiol Scand 2 (Suppl 8)
131 AGNER K 1943 Verdoperoxidase Adv Enzym 3 137
132 HUMBLE J G 1946 Quart J Med 15 299
133 GOMORI G 1953 J Histochem Cytochem 1 493
134 GLICK D 1954 Methods of Biochemical Analysis Interscience New York N Y
135 MCKINNEY G R S P MARTIN R W RUNDLES & R GREEN 1953 J Applied Physiol 5 335
136 WAGNER R & A YOURKE 1953 Arch Biochem Biophys 44 415
137 MCKINNEY G R 1953 Arch Biochem Biophys 46 246
138 LAVES W & K THOMA 1950 Arch path Anat Physiol Virchow's 318 74
139 SEABRA P 1951 Blood 6 949
140 ALLEN A 1952 Pathology W A D Anderson Ed Mosby St Louis Mo
141 LAJTHA L G F ELLIS & R OLIVER 1953 Brit J Cancer 7 401
142 WACHSTEIN M 1950 Proc Soc Exptl Biol Med 73 3061
143 WEISS L P 1953 Blood 8 249
144 KERBY G P 1952 Proc Soc Exptl Biol Med 81 129
145 KERBY G P & S N CHAUDURI 1953 J Lab Clin Med 41 632
146 HENDERSON M 1928 Anat Record 38 71
147 LEWIS W H 1934 Bull Johns Hopkins Hosp 49 29

some of our more recent experiments regarding the factors involved in producing an eosinophilia and possible interpretations from these findings

In 1889 Ehrlich and Lazarus pointed out that foreign materials such as parasitic extracts were chemotactic for eosinophils This finding has been confirmed in many experiments since then[56, 79, 86, 107, 109, 235] and Ringoen[174] concluded that foreign proteins are the chief factors responsible for the increased production of eosinophils It has been clearly demonstrated that repeated injections of a foreign protein or of an antigenic material will lead to a local and systemic increase in the number of eosinophils[6, 3, 34, 115, 154, 155] Furthermore clinicians have long been aware that eosinophils are in some way related to diseases of sensitization and respond specifically during such diseases[169] The number and proportion of eosinophils are greatly increased in patients afflicted with various types of allergy[18, 0, 3, 34, 61, 95, 99, 173, 155, 181, 183, 190, 17] with parasitic infection especially by nematodes[37, 33, 104, 107, 155, 17] and following anaphylactic reactions[34, 67, 63, 85, 95, 190, 193, 203] Ringoen[171] observed that every animal that survived an anaphylactic reaction exhibited an eosinophilia

Systemic or blood cellular changes have been extensively investigated and reported but there have been comparatively few studies in recent years of local responses of eosinophils to physiologically active materials This lack of data is due in part to the difficulty in obtaining quantitative estimations of cells outside the blood vessels as in connective tissue or in various organs of the body Webb[71] used fluid taken from slashed organs composed of tissue fluid cells plus blood from the minute blood vessels This method held a great deal of promise but it has not been popularly used Rebuck (in this monograph) and Dougherty[61] have developed excellent techniques for studying qualitatively cellular responses in tissue fluids but these techniques cannot be used for quantitative studies of the cells

Undoubtedly the most satisfactory method of overcoming the difficulty of quantitative measurements is to make use of the fluids which accumulate in various areas of the body as in the peritoneal cavity[14, 113, 1??, 157, 159, 160, 163, 194, 31, 33, 234, 235] in the pleural cavity[11, 7, 136] or in blisters[90, 91, 14, 15] in lymph[22, 186, 34] in sputum[224] in nasal secretions[179, 215] in aqueous and vitreous humors[119, 227] in urine and in stools

The fluid from the peritoneal space is an excellent medium for observing leukocytes and other cells especially following inflammatory changes produced there by hormones drugs and various other active substances With slight modifications the standard hematological techniques can be applied to peritoneal fluid

We began conducting a series of studies on the cells of the peritoneal cavity in an attempt to correlate the blood eosinophils with those found outside blood vessels In this manner we hoped to learn more about the physiology of these cells and the effect of certain hormones and drugs upon eosinophils This approach has proved so successful that it has been expanded to include a wider variety of materials having a physiological effect as well as a study of the actions and functions of other cells

One of the first studies we were able to perform with this technique was in the action of adrenal hormones upon eosinophils induced into the peritoneal

PHYSIOLOGICAL APPROACHES TO AN UNDERSTANDING OF THE FUNCTION OF EOSINOPHILS AND BASOPHILS

By R S Speirs*
Roscoe B Jackson Memorial Laboratory Bar Harbor Me

1 Eosinophils

The eosinophil cell of the blood has been a source of fascination to many people during the last 60 years Emil Schwarz published a paper reviewing eosinophils in 1914 listing over 2400 references Each succeeding year has seen the publication of hundreds of additional papers and at the present time it seems that no issue of a biological or medical journal would be complete without a reference to these cells[18 36 76 80 107 113 1 3 174 187]

There are probably a number of reasons for this tremendous interest in one of the less numerous cells of the blood This cell is certainly distinct in appearance and staining characteristics from all other cells of the body This distinction enables it to be readily seen in tissues as well as blood Most important, however is the fact that unlike most other cells of the blood relatively simple and rapid techniques are available for quantitative determinations in blood and tissue fluids These techniques are based upon (1) the ability of the eosinophil granules to stain with acid dyes and (2) the resistance of the cell to hypotonic solutions Some of the eosinophil diluents used have been briefly reviewed[204] and there have been many other papers describing the variations of the technique and the statistical treatment of the results to determine accuracy[4 15 19 29 58 65 131 166 2 0 237] There have also been two reports published in 1928 concerning the differentiation of eosinophils from pseudoeosinophils[189 200]

The techniques described have been extensively used in the clinic and in the research laboratory for a wide variety of studies Eosinophil levels or a change in these levels following specific stimuli have been used to assay adrenal cortical hormones[51 100 184 185 206] and ACTH[163 05] These cells have been used in the clinic to aid in the diagnosis of various conditions such as Addisonian or Cushing s disease[18 218 219] Loeffler s syndrome[135] certain malignant tumors[13 39 149 174] parasitic infections[1 3 14] benzol poisoning[1 3] and allergy[145 174] and to evaluate the doses of hormones needed in the treatment of certain diseases[18 52 199 243] Eosinophils have also been suggested as a means of detecting stress[103] of determining dangerous amounts of radiation[147] of evaluating responses to surgical trauma[49 126 151 177 196] and have been noted to accumulate in skin grafts which are about to slough off[179] *An increase in the number of eosinophils follows the termination of most infectious diseases and is used as a good prognostic sign*[1 3 174]

It is beyond the scope of this paper to attempt to review all the extensive literature concerning this cell Instead an attempt will be made to present

* Th in t g t r ported th p pe w s ppo ted by gr t f om th N t l C I t t t f th N t l I t t tes f H lth P bl H lth Se d f m th P bl H lth F d t for C er d Blood Pres Rese h Th th w l l k t xpres pp t to E. E Spe M g t Sm thbe g a d M ry D b ch f th s ggest d h lp

some of our more recent experiments regarding the factors involved in producing an eosinophilia and possible interpretations from these findings

In 1889 Ehrlich and Lazarus pointed out that foreign materials such as parasitic extracts were chemotactic for eosinophils This finding has been confirmed in many experiments since then[66, 79, 86, 107, 109, 235] and Kingoen[174] concluded that foreign proteins are the chief factors responsible for the increased production of eosinophils It has been clearly demonstrated that repeated injections of a foreign protein or of an antigenic material will lead to a local and systemic increase in the number of eosinophils[26, 30, 34, 115, 154, 155] Furthermore clinicians have long been aware that eosinophils are in some way related to diseases of sensitization and respond specifically during such diseases[169] The number and proportion of eosinophils are greatly increased in patients afflicted with various types of allergy[18, 20, 37, 34, 61, 85, 90, 123, 155, 181, 152, 190, 217] with parasitic infection especially by nematodes[30, 32, 104, 107, 155, 217] and following anaphylactic reactions[31, 67, 63, 85, 95, 190, 193, 223] Ringoen[174] observed that every animal that survived an anaphylactic reaction exhibited an eosinophilia

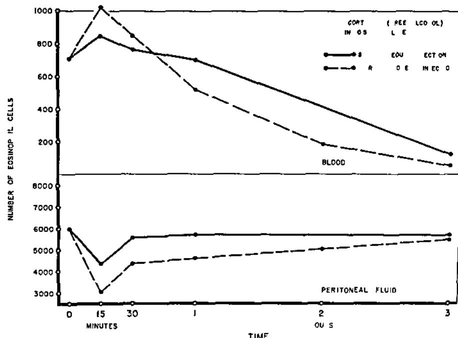
Systemic or blood cellular changes have been extensively investigated and reported but there have been comparatively few studies in recent years of local responses of eosinophils to physiologically active materials This lack of data is due in part to the difficulty in obtaining quantitative estimations of cells outside the blood vessels as in connective tissue or in various organs of the body Webb[31] used fluid taken from slashed organs composed of tissue fluid cells plus blood from the minute blood vessels This method held a great deal of promise but it has not been popularly used Rebuck (in this monograph) and Dougherty[60] have developed excellent techniques for studying qualitatively cellular responses in tissue fluids but these techniques cannot be used for quantitative studies of the cells

Undoubtedly the most satisfactory method of overcoming the difficulty of quantitative measurements is to make use of the fluids which accumulate in various areas of the body as in the peritoneal cavity[14, 119, 140, 157, 158, 160, 163, 194, 231, 3, 234, 235] in the pleural cavity[11, 47, 136] or in blisters[90, 91, 14, 15] in lymph[22, 186, 34] in sputum[74] in nasal secretions[179, 215] in aqueous and vitreous humors[119, 77] in urine and in stools

The fluid from the peritoneal space is an excellent medium for observing leukocytes and other cells especially following inflammatory changes produced there by hormones drugs and various other active substances With slight modifications the standard hematological techniques can be applied to peritoneal fluid

We began conducting a series of studies on the cells of the peritoneal cavity in an attempt to correlate the blood eosinophils with those found outside blood vessels In this manner we hoped to learn more about the physiology of these cells and the effect of certain hormones and drugs upon eosinophils This approach has proved so successful that it has been expanded to include a wider variety of materials having a physiological effect as well as a study of the actions and functions of other cells

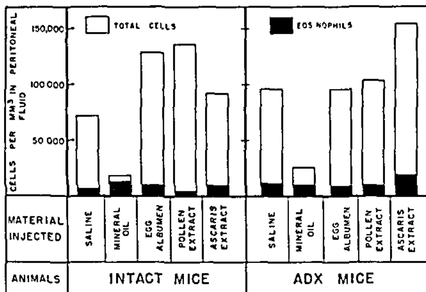
One of the first studies we were able to perform with this technique was in the action of adrenal hormones upon eosinophils induced into the peritoneal



FIGU 1 Comp so of th ffects f ub ta eo d t pe to al ı ct of 100 mı grams f co t sone upon e ph l th blood a d p to l fl d The t l l w oph l o t f ı t p t eally ı j ct d m l w du t a d l t on of th pe t n l fl d Th disapp d w th th ee h u (D ta from P e h g d Sp s 1953 [60])

fluid by repeated injections of horse serum By quantitatively determining the number of eosinophils in the blood and in the peritoneal fluid following the injection or following an implantation of a hard pellet of these hormones we found that the eosinophils decreased first in the blood and hours later, in the peritoneal fluid (see FIGURE 1) Cortisone produced essentially the same response whether it was given intraperitoneally or subcutaneously The disappearance of the eosinophils is explained by (1) the migration of the cells out of the peritoneal cavity and into the blood and (2) a local phagocytosis which goes on independently of the adrenal hormone s concentration[105, 157, 158] These results indicate that physiological doses of cortisone compound F and epinephrine do not have a direct lytic action upon eosinophils *in vivo* Similar results were obtained following histamine injections Accordingly we could not substantiate the hypothesis of Muehrcke[148] and Godlowski[87, 88, 89] that the adrenal cortical hormones produce a direct lytic action upon eosinophils It should also be noted that other investigators have not been able to repeat the experiments of these authors[10, 36, 50, 75, 76]

Our attention then turned to the factors involved in producing an increase in the number of eosinophils and of other cells in the peritoneal fluid In order to avoid the tremendous fluctuations of blood cells brought about by stress produced by handling[208] adrenalectomized animals were used as well as intact animals Genetic factors and sex differences known to have marked influences on physiological responses[41] were controlled in these experiments by using



F URE 2 Compa ison f th ll la pouse i th pe t l fl id f llow g gl l traperiton l jec tion f io bst nc s.

hybrid BBF_1 male mice The techniques used in these studies are to be reported elsewhere Chamber counts[204] were made for (1) the total number of cells (2) eosinophils and (3) mast cells In addition films were made and stained with Wright Giemsa blood stains and differential counts were performed In some cases the leukocytes were clumped together making an accurate total count impossible However this clumping did not occur when the eosinophil diluent was used and this count was considered to be reasonably accurate[180]

FIGURE 2 shows the total number of cells and eosinophils found in peritoneal fluid of male BBF_1 mice 48 hours following a single injection of various materials The saline injected animals listed in the first column on the left contained cell numbers which were not significantly different from uninjected mice (not shown) Single injections of egg albumen pollen extract (Mixed Ragweed Pollen Extract Abbott Laboratories) ascaris extract and numerous other materials produced an increase in the peritoneal cells due primarily to an increased concentration of polymorphonuclear neutrophils This effect is similar to the response which has been reported in other animals[14, 107, 193, 194, 231] The injection of mineral oil caused an over all increase in the amount of fluid in the peritoneal cavity However there was a depression of the total number of cells per cubic millimeter This reduction was partly due to the unabsorbed mineral oil which was taken up in our pipets along with the peritoneal fluid The differential counts made from these animals showed a marked increase in the proportion of polymorphonuclear neutrophils present

It may be further seen in FIGURE 2 that there is not a marked difference in

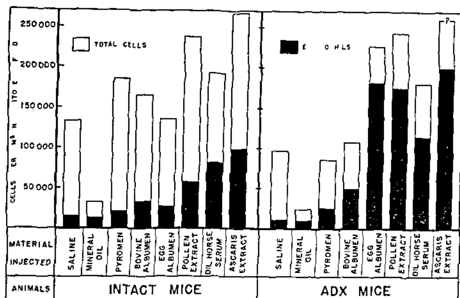


FIGURE 3 Comparison of th ll l po es the p t 1fl d foll w g m lt j l t ap t l j t of b t s Ad l t my lt di h gh r o ph l t imal e v g t g s b ta c b t t mal v g l m l l py m Th t tal mb f ll r t t te be ea clump g f the ll m d ti g d ff lt Th l m g wa t bse ed th ph l d l e t

the response of adrenalectomized mice on the right hand side and the intact mice on the left hand side Thus single injections of antigenic material do not produce marked increases in the total number of cells or in the number of eosinophils in the peritoneal fluid 48 hours after an intraperitoneal injection Oil injections on the other hand produced a marked decrease in the number of mast cells present in the peritoneal cavity

FIGURE 3 shows the response of peritoneal cells to repeated injections of various substances These animals were injected on Monday Tuesday and Wednesday for the first week and every Monday for the following three weeks Determinations of the peritoneal cells were made 48 hours following the last injection It may be seen that with the exception of the mineral oil repeated injections of these materials caused an increase in the total number of cells in the peritoneal fluid There was an increase in the number of eosinophils in all groups but the animals receiving pollen extract horse serum (diluted 1 100 with saline) and ascaris extract had the most striking increases Furthermore there was a marked difference in the eosinophil response of the adrenalectomized mice shown on the right over the intact animals This difference was due to a marked increase in the proportion and absolute number of eosinophils present in the adrenalectomized mice

This increase in eosinophils in intact and adrenalectomized animals is a specific reaction to the reinjection of specific antigenic material The same antigen must be injected repeatedly at wide intervals in order to obtain the eosinophilia injections of different substances do not give the response [6, 35, 116]

Furthermore various amino acids tried did not produce the response but the polypeptides did [6,104,193]

The eosinophil response reported here in the adrenalectomized mice is reminiscent of the allergic responses found in the clinic [71,193] For example Kingoen (1938)[174] has pointed out that a blood eosinophilia is generally recognized as a constant and definite feature of allergy

The eosinophil is the only cell which is known to respond specifically during states of hypersensitivity,[1,3,169] and it has been shown that these cells are specifically attracted to the shock tissues [19,191] Eriksson Ehr[74] noted that in a study of 500 cases of allergies there was observed a consistent tendency to lymphocytosis and eosinophilia Furthermore there was a disturbance of the mineral or carbohydrate metabolism and other indications of a hypofunctioning adrenal cortex Similar comparisons have been made by Rose[183] who noted that in addition to a decreased adrenal cortical secretion there is a disturbed histamine metabolism and similar effects on antibody production and reticuloendothelial activity Furthermore he points out that the mouse is an excellent experimental animal for studying the effects of reversible hypersensitivity brought about by cortisone The sensitized adrenalectomized mouse accordingly resembles the allergic patient in many ways and might well be used as one approach in the study of this condition

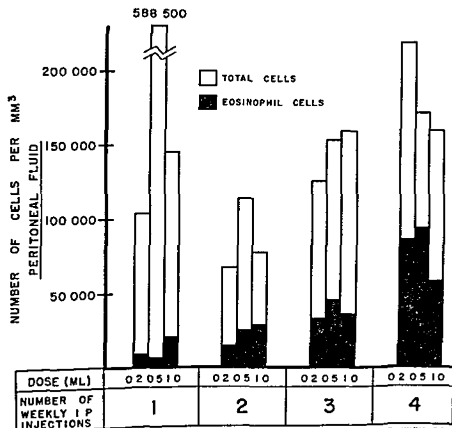
The relationship to allergy is illustrated even further in FIGURE 4 A group of 36 mice was adrenalectomized and then given weekly injections of a ragweed pollen extract Each week the total cellularity and number of eosinophil were determined 48 hours following the last injection It may be seen that there is a progressive increase in the number and percentage of peritoneal eosinophils following each injection of the pollen

The peritoneal cavity is an excellent site for the study of local cellular responses following the addition of an adjuvant and various other agents to the material injected For example various oils have been found to reduce the total number of cells per cubic millimeter of peritoneal fluid but there is a marked increase in the total amount of fluid present Water in oil emulsions of antigen are known to produce an enhanced effect upon the antibody production presumably by slowing the absorption rate of the antigen [81] It is of interest therefore to know whether the oil inhibits or changes the local cellular response to antigen injections However as can be seen in FIGURE 5 the addition of sesame oil to egg albumen or pollen extract did not significantly change the number of eosinophils per cubic millimeter of peritoneal fluid In these injections as well as in the other injections of oil the mast cells were strikingly reduced Further studies with other adjuvants and with measurements of total fluid volume and differential cell counts can be easily undertaken

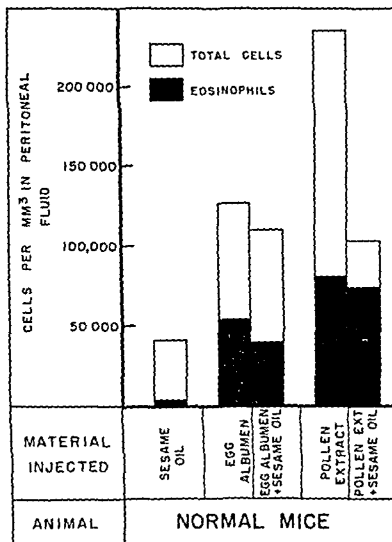
The effect of the various antigenic materials upon eosinophils may be due to (1) the antigen *per se* and (2) the syndrome which follows the antigen antibody reaction There is a great deal of data available indicating that the antigen itself does not have the capacity to produce an eosinophilia It was shown in FIGURE 2 that a single injection of an antigen does not produce a local eosinophilia We have given up to 30 000 pollen units in a single injection without producing an eosinophilia in mice Furthermore multiple injections of the

antigen given simultaneously will not produce this effect For example Campbell in 1942[33] performed as many as 40 intramuscular injections in a single nonsensitized guinea pig without obtaining an eosinophilia The antigen has to be injected into an animal that had been previously treated with the same material at an earlier date in order for an eosinophilia to occur Herrick[101] noted that daily intraperitoneal injections of antigen produced much less eosinophilia than antigen injected at 5 to 25 day intervals The eosinophilia obtained does not appear to be proportional to the quantity of antigen injected FIGURE 6 shows the results obtained following 0 2 ml injections of three dilutions of a pollen extract It can be seen that there was no great reduction in the eosinophil response as the pollen was diluted with 10 or 100 parts of saline Similar results were obtained with various injections of egg albumen, horse serum, and an ascaris extract

These experiments indicate that it is not the antigen *per se*, but the reaction of the sensitized organism to that specific antigen that is responsible for the eosinophilia

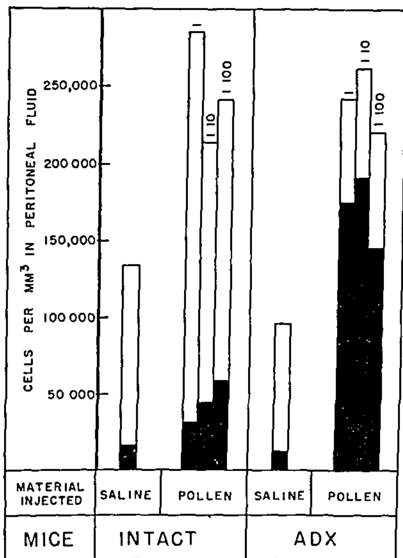


F GURE 4 Eff ct f weekly ı trape to l ı ct f poll t t th ll la ty f th pe to al fl d of drenal ctomized m Followı g a h ject th w ease in th umb d p port f eos ophils



F ure 5 Eff t f sesame [illegible] ll la ty f th pe ton l fl d p od ed b m lt pl nj cti f nt g Th ject n f sesam o l d d t b g th eos ph l po se to th t ge

Following the introduction of antigen into a sensitized animal a number of active substances are known to be released especially if anaphylactic reactions occur These materials including histamine[41] heparin[113, 120] and possibly choline[82] hyaluronidase[68] and other active substances[35, 135] are responsible for many of the symptoms observed It is possible that one of these substances may be responsible for the eosinophilia which follows this reaction[180, 190, 191] The various antigens may thus have some common denominator *i e* some material that would cause an attraction of eosinophils to the site of antigen



ADX MICE MAINTAINED ON BA 10386

Fig. 6 [illegible]

antibody reaction This common factor would not be released except by the specific antigen to which the animal was sensitized A study was therefore undertaken of the local eosinophilia produced by various substances believed to be released during or immediately following the antibody antigen reaction The following is a preliminary report of our results

The first substance considered was histamine, which is present in almost every mammalian tissue.[illegible] Histamine is released under various physiological and pathological conditions[illegible] and it produces, in the free state, very striking responses in blood[illegible] and has been reported to have a chemotactic effect upon eosinophils.[illegible] Furthermore, histamine metabolism appears to be grossly disturbed in common forms of allergy.[illegible] It increases in the tissues following adrenalectomy[illegible] and has been shown to be released by sensitized blood cells following the *in vitro* addition of an antigen such as pollen.[illegible] Histamine has been clearly identified in blood cells[illegible] and is *definitely in higher proportion in the granulocytes than in* the lymphocytes and monocytes.[illegible] It has been postulated that eosinophils may be the chief carriers of histamine in man.[illegible] However, it is more likely that histamine and eosinophils are both related to the allergic state but are quite independent of each other.[illegible] Nevertheless, Vaughn in a recent review[illegible] states that "the existence of an eosinophilia of the blood may indicate that somewhere in the body histamine or some similar toxic substance is being set free in abnormal quantities." *In fact*, Kovacs[illegible] and Vercauteran[illegible] have suggested in their reports that the eosinophils appear to contain antihistamine activity. This inference could be incorporated into a very plausible hypothesis on the function of these cells. However, further work is necessary.

It seemed to us that the local peritoneal responses of eosinophils to this material might give a clue to the eosinotactic nature of histamine. The data obtained from these experiments are shown in FIGURE 7. A single injection of histamine produced first a decrease in the total cellularity, undoubtedly due in part to a dilution effect of the vehicle. Within six hours, the total number of *cells began to increase while the eosinophils remain unchanged.* Within 24 hours there is a slight increase in both total cells and eosinophils. This rather slow reaction does not suggest that histamine is a direct eosinophil tactic substance, but rather that it is an over-all inflammatory stimulant involving all cells.[illegible]

Injections of choline chloride and hyaluronidase into normal mice failed to produce a marked increase in the number of local eosinophils when deter*mined 24 hours after the injection.*

Heparin, on the other hand, gave quite a different reaction. This material had first been freed of possible bacterial contamination by passing it through a Seitz asbestos filter. When injected, it produced a very marked eosinophilia (see FIGURES 8 and 9). It has been recently discovered, however, that this effect is due to asbestos fibers present in the filtrate which were inadvertently injected into the animals. If the heparin is filtered through fritted glass, this *reaction does not occur. Therefore, heparin, as well as hyaluronidase, hista*mine, and choline, do not seem to be responsible for the eosinophilia which occurs following antigen-antibody reactions.

Thus our experiments to date have not yielded a single physiological material responsible for the observed allergic eosinophilia. It would be of interest, however, to inject intraperitoneally the antigen or antibody and various combinations of the antigen and antibody or the actual precipitin, to determine the

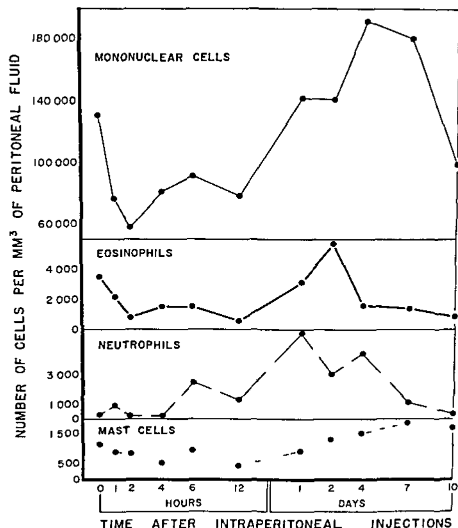
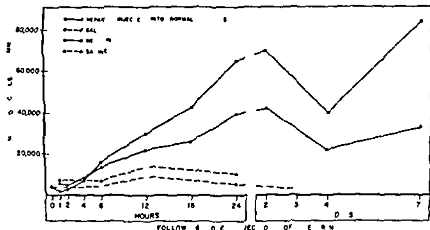


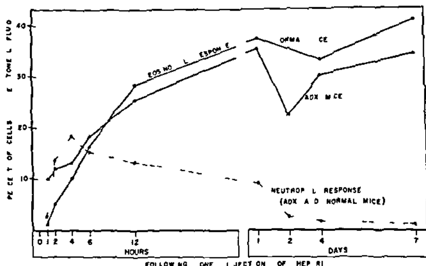
FIGURE 7 Th ffect f a gl j ct o f 0 2 mgm f h tam d p h phat th umb f ll th p rito eal fl d f ormal m

cellular responses to each *Experiments of this type would aid in understanding the local cellular changes when antigen and antibody combine*

Methods of producing eosinophils in the peritoneal fluid for harvesting Local eosinophil concentrations can be produced by a number of different techniques In mice given a course of weekly injections of pollen extract or some other antigenic material local eosinophilias of 30 to 50 per cent occur The proportion of these cells may be increased greatly by adrenalectomy The dose of material used is relatively small approximately 1 milligram divided into six or eight injections is usually sufficient to produce this effect The eosinophils begin to accumulate following the second or third injection



F g 8 Effect f Se tz filt red h parı po the n mbe f eos ph l i the pe t l c ty Th se l eo l ph l w d to bestos fibers d tently jected l g w th th h par n



F g 9 Th ch g th p po t f eo ph l d n t ph l th pe to l d d f ll ng th fecti f Se tz filt ed h pa Th h g eos ph l w d t be tos fibers d rt tly j t d 10 g w th th h p

Very recently it was found in our laboratory that substances which had been Seitz filtered produced a local eosinophilia within 24 hours This response is exemplified by FIGURES 8 and 9 It may be seen that the number of eosinophils increased tremendously in normal and adrenalectomized mice within two to six hours following the injection of heparin which had been Seitz filtered One hour after the injection there were approximately 1000 cells per cu mm in normal mice and 5 000 in adrenalectomized mice Twenty four hours later

the number of eosinophils was 64,983 in intact mice, and 39 063 in adrenalectomized mice

This accumulation of eosinophils was found to be caused by the injection of small fibers from filter pads The eosinotactic substance of these filters is not water soluble and is not removed by ashing It appears that the actual asbestos fibers attract eosinophils and oddly enough other similar or irritating substances such as magnesium carbonate ground glass paper and cotton fibers do not produce this response At the present time, the response seems to be specific to the asbestos fibers found in the filter pad This appears to be the first time that any substance with this direct rapid action has been reported

To obtain the accumulation of eosinophils by the asbestos fibers a suspension of 0 1 per cent fibers in saline solution is obtained The fibers may be ashed first the soluble material washed away and the remaining suspension sterilized by boiling Injections of 0 2 to 1 0 ml are made into the peritoneal cavity and the cells are harvested after 24 hours The number of eosinophils in the peritoneal cavity remains high for weeks following a single injection However it should be noted that stress ACTH or adrenal cortical hormone injections will inhibit this reaction or cause a disappearance of the eosinophils after the accumulation has occurred

2 Basophil Cells

Probably one of the best examples of how much or how little is known about basophil cells of the blood is to be found in Michels paper in Hal Downey s *Handbook of Hematology*[14] Michels listed 25 different hypotheses concerning the function of basophil and mast cells These theories include everything from degenerating connective tissue elements to immature pigment cells So little is known about these cells that often the wildest speculation will pass unchallenged In 1941 Doan and Reinhardt[57] observed that the experimental approach has yielded little of significance to the problem presented by basophil granulated cells and of all the blood cells least is known about them Undoubtedly the most important reasons for the failure of the experimental approach are a lack of a quantitative method of studying these cells and a lack of experimental methods of producing measurable changes in these cells Both of these factors have been recently overcome[144, 170] and it is to be expected that a rapid advancement in knowledge of these cells will be seen in the near future

Mast cells and basophils were first described by Ehrlich in 1877 and 1879 He recognized that these two cells had identical basophilic metachromatic staining reactions but otherwise they appeared to have little in common Some of these differences were summarized in 1941 by Doan and Reinhardt[57] who pointed out that the basophil is produced by the bone marrow and that the entire range of maturation can be seen there Moreover these cells are relatively small show ameboid motility and contain granules which vary markedly in size and are usually water soluble They retain their leukocyte characteristics even after migration into tissues

The tissue mast cells on the other hand develop *in situ* They are larger

cell have uniformly large coarse less-soluble granules and they show little or no ameboid motility There are also other differences in the shape and structure of the nucleus and staining reaction of these two cells [12, 142]

Like Doan and Reinhardt most workers in this field have been careful to note that there are marked differences between the two types of basophil staining cells [11, 14] In recent years however many of the so-called differences between the two types of cells are beginning to be resolved For example tissue culture studies of the mast cells by Paff and Bloom (1949) [159] and Zitcer (1953) [4] have indicated that these cells are quite active and vary tremendously in size and shape The granulation of the cells varies in a somewhat cyclic manner Young cells appear to have fine granules older cells have larger coarser granules

There are many indications of a functional correlation between the two types of cells [40] For example the response of mast cells and basophils to stress and cortisone treatment appears to be similar Mast cells have been reported to disintegrate and disappear following stress[10] or cortisone injections[7, 28, 81, 151] and just recently Code[40] reported that similar decreases in blood basophils occurred following oral cortisone administration The disappearance of basophils during acute inflammation or stress has been known for many years [142, 148] Although there does not appear to be much disagreement concerning the action of stress on these cells not all workers have been able to observe a cortisone effect on them [55, 196] and more work of a quantitative nature is needed It should be pointed out that the peritoneal fluid is an excellent site to study changes in number of mast cells and this approach is being actively investigated (personal communication from Doctors G M Higgins and J Padawer) Preliminary experiments in our laboratory have indicated that the mast cells are still present unchanged in number in the peritoneal fluid at 4 and 24 hours following a single injection of 20 micrograms of cortisone acetate It may be that larger doses of cortisone are necessary to get this effect and that the action may be pharmacological rather than physiological Adrenalectomy on the other hand does decrease the number of mast cells found in the peritoneal fluid For example the mean mast cell count in 140 intact mice was 549 per cu mm In 142 adrenalectomized mice receiving the same treatment the count was only 253 per cu mm Thus there does not appear to be any doubt but that the mast cells and possibly the basophils are related in some way to the adrenal but the exact nature of this relationship is not known

Holmgren and Wilander[104] first noted that there was a positive relation between the amount of heparin that can be extracted from the tissue and the number of mast cells in that tissue Since then there has been a tremendous amount of work and speculation concerning the role of mast cells in producing and storing heparin [5, 79, 138, 207, 38, 214] It is interesting to note that in 1946 Jorpes[113] wrote that there is no doubt whatsoever about the nature of the granular substances of the mast cells It is heparin Likewise Friberg and his co workers[8] concluded after reviewing the literature that heparin is stored and probably produced by the mast cells This conclusion has been emphasized recently by reports of Jorpes[114] and Asboe Hansen[9] that an uptake of S^{35} has

been demonstrated by mast cells Furthermore extracts of some mast cell tumors[15, 159] and pathological tissue containing abnormal quantities of mast cells[67] have been shown to contain extremely large amounts of heparin

Heparin will stain metachromatically but other substances such as hyaluronic acid sulfuric acid ester *etc* will also stain in this way[133, 131] Although mast cells are commonly found in areas which are rich in hyaluronic acid[7] this material is not demonstrable as such in the mast cell[09, 40] Schoch[195] however has suggested that a hyaluronidase inhibitor may be present Asboe Hansen[7] has postulated that the mast cells secrete hyaluronic acid and the materials staining in the mast cells are precursors which resemble heparin in their staining reaction In any case hyaluronidase has no effect on the metachromatic staining of these cells and neither does ribonuclease or desoxyribonuclease[240, 45]

Sundberg[09] has demonstrated how easy it is to be misled in determining the anticoagulant activity of tissue extracts She found that there was a marked difference in the activity depending upon whether the extraction was done with water or sodium chloride The concentration of the salt was found to prolong the coagulation time markedly and must therefore be carefully controlled Moreover she pointed out that the umbilical cord contained many mast cells while the placenta had very few yet extracts of the placenta often contain as much or even more heparin like material than do similar extracts of the cord With this fact in mind many of the extraction procedures reported for heparin should be reconsidered to determine if the authors could have been misled by the salt concentration of their extracts

The basophils of the blood also have recently been shown to be somewhat correlated with extractable anticoagulant activity In chronic myelogenous leukemia as well as in mast cell leukemia anticoagulant substances have been observed[138, 209, 216]

During the last 10 years there have been a number of interesting experiments concerning heparin and lipid metabolism and recently it was suggested that heparin may be more important in lipoprotein metabolism than in blood coagulation[10, 08] This plasma clearing effect of heparin was first reported by Hahn[96] The action is very rapid and involves a disappearance of chylomicrons from the blood and spleen[1, 2, 79, 95, 210, 211, 21, 236] and a change from low density to high density lipoproteins[30, 93, 10] Ringoen in his review[174] indicated that mast cells have been related to fat metabolism and according to some authors they may even transform into fat cells With these observations in mind it would be of interest to determine whether lipids have any effect on mast cells of the peritoneal fluid In a group of 50 normal and adrenalectomized mice we found that the mast cells almost completely disappeared from the peritoneal fluid 24 hours following an intraperitoneal injection of oil or of oil plus various antigens This effect is very striking and it indicates that lipids cause a disappearance of mast cells Further work is being carried out to characterize this response and to determine if the mast cells also disappear in the omentum

The evidence for heparin production and storage by mast cells and basophils is accordingly very indicative The definitive experiments proving this re

lationship however and the mechanisms of storage and release have yet to be reported

Histamine has also been associated with tissue mast cells in normal and inflamed tissues and in mast cell tumors[66, 171, 172] Although it has been demonstrated that heparin will prevent the release of histamine[66] both substances are released during anaphylactic reactions[41, 217] and following injections of histamine liberators[102, 133b] Basophils apparently contain large amounts of histamine per cell Graham[66] estimated that they may contain up to 50 times the amount of histamine (per cell) found in other blood cells Further quantitative studies are necessary

Michels[128] pointed out that animals with many blood basophils often have few tissue mast cells and vice versa The mouse for example has practically no blood basophils yet there are many mast cells found in the peritoneal fluid The reverse is true of the rabbit This relationship is exemplified even further by the observation that the mast cells in the peritoneal fluids and the basophils both greatly increase in myelogenous leukemia[111, 155] The two cells accordingly appear to be related in many ways and it is *therefore possible that the blood* basophils are juvenile forms of the tissue mast cells This relationship however remains to be demonstrated if it really exists By applying tissue culture techniques this problem will undoubtedly be solved

The techniques used for the study of basophil cells are very important This fact has been demonstrated by Graf and Swensson[8] who found that May Gruenwald Giemsa stain caused a washing out of many of the basophil granules and that toluidine blue stained blood smears gave a higher basophil count Moreover these investigators observed that a perforating injury to a vein elicits a local increase of basophils in the differential count which occurred within 30 minutes and lasted up to 24 hours This local accumulation of cells can be very misleading and may explain many of the reported basophilias obtained following multiple injections of various materials It is also possible that the basophilia following intense irradiation[4, 72, 147] may also be due to injury of the veins

Summary

We have attempted to describe a physiological approach to the study of the function of leukocytes This approach takes advantage of the accumulation of cell laden fluid in the peritoneal cavity It is a method of studying leukocytes outside the blood vessels where they normally carry on many of their important functions

We have demonstrated that changes in the number and type of cells in the peritoneal fluid of mice can be obtained by intraperitoneal injections of various hormones drugs and other physiologically active substances For example *subcutaneous and intraperitoneal injections of adrenal cortical hormones produce* a marked decrease in the number of eosinophils in the blood and later in the peritoneal fluid It has been demonstrated that these hormones do not act directly upon the eosinophils or mast cells in physiological doses and their effect cannot be explained on the basis of a direct lytic action These peri

20 BICKEL, G 1950 Value of eosinophilia in the diagnosis of allergic states (French) Acta Allergolozica Suppl. 1 57

21 BIERICH R 1922 Über die Beteiligung des Bindgewebes bei der experimentellen Krebsbildung Arch path Anat Physiol Virchow s 239 1

22 BIERMAN H R R L BYRON JR K H KELLY R S GILFILLAN L P WHITE N E FREEMAN & N L PETRAKIS 1953 The characteristics of thoracic duct lymph in man J Clin Invest 32 637–649

23 BIERMAN H R R L BYRON K H KELLY F CORDES L P WHITE & A LITTMAN 1953 The influence of intra arterial administration of histamine upon the circulating leukocytes of man Blood 8 315–323

24 BIERMAN H R K H KELLY N L PETRAKIS F CORDES M FOSTER & E A LOSE 1951 The effect of intravenous histamine administration on the level of the white blood count in the peripheral blood Blood 6(10) 926–935

25 BIFANI I & P CASOL 1951 Contributo sperimentale allo studio dei rapporti tra eparina e mastzellan di Ehrlich (Experimental contribution to the study of relations between heparin and the mast cells of Ehrlich) Sperimentale 101 35–47

26 BIGGART J H 1932 Some observations on the eosinophile cell J Path Bact 35 799–816

27 BINE R 1918 Pleural eosinophilia Am J Med Sci 155 579

28 BLOOM F 1952 Effect of cortisone on mast cell tumors (Mastocytoma) of the dog Proc Soc Exptl Biol Med 79 651–654

29 BONNER C D 1952 Eosinophil levels as an index of adrenal responsiveness factors that affect value of eosinophil counts J Am Med Assoc 148 634–637

30 BOYLE E J H BRAGDON & R K BROWN 1952 Role of heparin in in vitro production of alpha lipo-proteins in human plasma Proc Soc Exptl Biol Med 81 475

31 Reference cancelled

32 CAMPBELL A C P A M DRENNAN & T RETTIE 1935 The relationship of the eosinophile leukocyte to allergy and anaphylaxis J Path Bact 40 537

33 CAMPBELL D H 1942 Experimental eosinophilia with Keratin from Ascaris suum and other sources J Infectious Diseases 71 270–276

34 CAMPBELL D H 1943 Relationship of eosinophil response to factors involved in anaphylaxis J Infectious Diseases 72 42–48

35 CAMPBELL D H & P A NICOLL 1940 Studies on in vitro anaphylaxis and release of an active non histamine material from sensitized guinea pig lung J Immunol 39 103

36 CAPE R D T J W THOMAS & R A PALMER. 1952 The effect of adrenal steroids on eosinophils Can Med Assoc J 66 441–444

37 CAPE R D T 1952 Eosinophil cells of the blood A general review Edinburgh Med J 59 374–384

38 CAVALLERO C & C BRACCINI 1951 Effect of cortisone on the mast cells of the rat Proc Soc Exptl Biol Med 78 141–143

39 CLAUDE A 1929 Eosinophils and malignant tumors Liége Medical 22 793–802

40 CODE C F R G MITCHELL & J C KENNEDY 1954 The effect of cortisone on the number of circulating basophils and eosinophils Is there a relationship between these cells? Proc Staff Meetings Mayo Clinic 29 200–204

41 CODE C F & H R ING 1937 The isolation of histamine from the white cell layer of centrifuged rabbit blood J Physiol 90 501–510

42 CODE C F 1952 Histamine in blood Physiol Revs 32 47–65

43 CODE C F 1937 Histamine like activity of white blood cells J Physiol 90 485–500

44 CODE C F 1953 The histamine content of white blood cells Blood Cells and Plasma Proteins J L Tullis Ed Academic Press New York N Y

45 CODE C F P B DEWS & G M HIGGINS 1953 The relationship of the adrenal gland to concentration of histamine and number of leucocytes in the blood of rabbits J Physiol 121 487–491

46 CODE C F & A D MCDONALD 1937 The histamine like activity of blood Lancet 2 730–733

47 COMPTON A S 1952 A cytochemical and cytological study of the connective tissue mast cell Am J Anat 91 301–329

48 COOKE R A 1932 Infective asthma indication of its allergic nature Am Soc Med Sci 183 309

49 COPPINGER W R & M G GOLDNER 1950 The eosinophil response to surgical trauma Surgery 28 75

50 Coste F F Delbarre & G Basset 1952 Essais de mise en évidence dans le sang des suettes traités par la cortisone d une substance anti inflammatoire Rev Rhum 19 327
51 Coste F F Laurent & F Delbarre 1951 L eosinophilie du rat surrénalectomisé et ses variations sous l influense du stress et de divers stéroïdes Compt rend soc biol 145 838
52 Coste F F Delbarre & G Basset 1950 Intérêt de la surveillance de l eosinophilie au cours des traitements par la corticostimuline hypophysaire (Value of surveillance of eosinophils during treatment with ACTH) Semaine hôpit Paris 28 3043-3047
53 Demole M 1953 Eosinophilie et parasitoses Gastroenterologia 79 44
54 Denison M F & M X Zarrow 1954 Eosinophils of blood during prolonged exposure to cold and chronic administration of cortisone acetate Proc Soc Exptl Biol Med 85 433
55 Devitt J F W J Pirozynski & P B Samuels 1953 Mast cell resistance to hormonal influence Proc Soc Exptl Biol Med 83 335-33
56 Dewitt W B 1953 Influence of *Schistosoma mansoni* infections on the eosinophil level of adrenalectomized mice Exptl Parasitol 2 358-365
57 Doan C A & H L Reinhart 1941 Basophil granulocyte basophil cytosis and myeloid leukemia basophil and mixed granule types experimental clinical and pathological study with report of new syndrome Am J Clin Path 11 1 39
58 Donato K A & M M Strumia 1952 An exact method for the chamber count of eosinophils in capillary blood and its application to the study of the diurnal cycle Blood 7 10 0-1079
59 Dougherty T F 1950 The protective role of adrenal cortical secretion in the hypersensitive state Am Assoc Advancement Sci Symposium on the Adrenal Cortex New York N Y 78
60 Dougherty T F 1952 Studies of the antiphlogistic and antibody suppressing functions of the pituitary adrenocortical secretions Rec Prog Hormone Res 7 307 330
61 Dragsted R J 1953 The circulating eosinophil cells in hayfever patients in relation to specific desensitization Acta allerg 6 103-106
62 Dragstedt C A 1941 Anaphylaxis Physiol Revs 21 563-587
63 Dragstedt C A M Ramirez de Arellano & A H Lawton 1940 Relationship of histamine to anaphylaxis in rabbit Science 91 617-618
64 Dragstedt C A J A Wells & M Rocha e Silva 1942 Inhibitory effect of heparin upon histamine release by trypsin antigen and proteose Proc Soc Exptl Biol Med 51 191 192
65 Dunger R 1910 Ein einfache Methode der Zahlung der eosinophilen Leukocyten und der praktische Wert dieser Untersuchungen Münch med Wochschr 37 1942
66 Eckert D & G Totterman 1950 Histamine content of white blood corpuscles with special reference to eosinophils Scand J Clin Lab Invest 2 59-61
67 Ehrich W E J Seifter H E Alburn & A J Begany 1949 Heparin and heparinocytes in elephantiasis Scroti Proc Soc Exptl Biol Med 70 183-184
68 Ehrich W F 1953 Histamine in mast cells Science 118 603
69 Ehrlich P 1891 Farbenanalytische Untersuchungen zur Histologie und Klinik des Blutes Hirschwald Berlin Germany
70 Ehrlich P 1879 Uber die specifischen Granulationen des Blutes Arch Anat u Physiol 571-579
71 Ehrlich P 1877 Beitrage zur Kenntnis der Anilinfarbungen und ihrer Verwendung in der mikroskopischen Technik Arch mikroskop Anat Entwicklungsmech 13 263
72 Ehrlich P & A Lazarus 1898 Die Anaemia Holder Wien Austria
73 Elliott A & R Young 1931 Myelogenous leukemia Med Clinics N Amer 15 569
74 Ericksson Lihr Z 1952 The function of the suprarenal cortex in allergic diseases Bull Schweiz Akad Med Wiss 8 174 179
75 Essellier A F P Jeanneret E Kopp & L Morandi 1954 Evidence against destruction of eosinophils by glucocorticoid as shown by *in vitro* experiments Endocrinology 64 411 419
76 Essellier A F R L Jeanneret & L Morandi 1954 The mechanism of glucocorticoid eosinopenia Contribution to the physiology of eosinophil granulocytes Blood 9 531 549
77 Fabris A 1927 Mastocitomi cutan da atrame Path logica 19 157
78 Farr P S 1951 Experiments on the fate of the lymphocyte Anat Record 109 515-534

79 FLYNN J E & R W COON 1952 Fundamentals of blood clotting Ann Rev Physiol 14 205–234
80 FREUCHEN I B 1930 Einleitende Versuche zur Feststellung des Verhaltens der eosinophilen Zellen bei antistoffbildenden Processen Acta Path Microbiol Scand Suppl 3 123–132
81 FREUND J J CASALS & E P HOSMER 1938 Sensitization and antibody formation after injection of tubercle bacilli and paraffin oil Proc Soc Exptl Biol Med 37 509–513
82 FRIBERG U W GRAF & B ABERG 1951 On the histochemistry of the mast cells Acta Path Microbiol Scand 29 197–207
83 *FULTON G P & F L MAYNARD 1953 Effect of cortisone on tissue mast cells in the* hamster cheek pouch Proc Soc Exptl Biol Med 84 259–260
84 GADDUM J H 1951 Metabolism of histamine Brit M S 987
85 DE GAETANI G F 1938 L eosinofilia da elminti tassegna sintetica (Eosinophilia due to helminthiasis review of literature) Arch ital med sper 2 567–597
86 GIFFIN H Z 1919 Persistent eosinophilia with hyperleukocytosis and splenomegaly Am S M Sci 158 618
87 GODLOWSKI Z Z 1951 Prevention of hormonal eosinopenia and lymphopenia by inhibition of clotting in blood preliminary report Brit Med J 4711 854–855
88 GODLOWSKI Z Z 1953 Enzymatic Concept of Anaphylaxis and Allergy and the Role of Eo inophils in Anaphylactic Reactions Related to Hormonal Alterations Livingstone Edinburgh Scotland
89 GODLOWSKI Z Z 1952 The fate of eosinophils in hormonally induced eosinopenia and its significance J Endocrinol 8 102–125
90 GOODMAN M H 1941 The artificial blister in the study of eosinophils with particular reference to dermatiti herpetiformis J Investigative Dermatol 4 349–364
91 GRACE A W 1947 Significance of eosinophilia in blister fluids and peripheral blood in *Pemphigus vulgaris* J Investigative Dermatol 8 339–343
92 GRAF W & A SWENSSON 1950 Experimental investigations on local changes in the white blood cell picture following perforating injury to blood vessels (veins) J Path Bact 62 419–427
93 GRAHAM D M T P LYON J W GOEMAN H B JONES A YANKLEY S SIMONTON & S WHITE 1951 Blood lipids and human atherosclerosis II The influence of heparin upon lipoprotein metaboli m Circulation 4 666
94 GRAHAM H T F WHEELWRIGHT H H PARISH JR A R MARKS & O H LOWRY 1952 Distribution of histamine among blood elements Federation Proc 11 350
95 GROSSMAN M I & I H STRUB 1954 Effect of heparin on fate of intravenously administered fat emulsions in rats Proc Soc Exptl Biol Med 85 356–359
96 HAHN P F 1943 Abolishment of alimentary lipemia following injection of heparin Science 98 19
97 HAJOS K 1928 Bietrage zur Eosinophilie frage III Uber den Zusammenhang zwischen der Bluteosinophile und der Immunkorperbildung Z ges exptl Med 59 389
98 HAJOS K 1928 Beitrage zur Eosinophilie frage II Mitteilung Die Verteilung der eosinophilen Zellen nach Proteininjektions und in anaphylaktisieren Neerschwein then Z ges exptl Med 59 383
99 HAJOS K & R MAZGON 1929 Beitrage zur Eosinophilie frage IV Die Desensibilisierungseosinophilie Z ges exptl Med 68 767
100 HALBERG F 1952 Some correlations bet veen chemical structure and maximal eosinopenia in adrenalectomized and hypophysectomized mi e J Pharmacol Exptl Therap 106 135–140
101 HALPERN N & S A BENOS 1951 Action de l histamine sur l eosinophilie sanguine chez l animal et l homme (The effect of histamine on eosinophil in man and animals) Compt rend soc biol 145 31–37
102 HAVEL R J & E BOYLE 1954 Production of lipemia clearing factor during anaphylactoid shock Proc Soc Exptl Biol Med 85 468–472
103 HEMING A M SAX & D HOLTKAMP 1952 Technique for detecting the pharmacodynamically induced alarm r acti n u ing the eosinopenic response of intact rats J Pharmacol Exptl Therap 106 271 276
104 HERRICK W W 1913 Experimental eos nophil a with an extract of an animal para site its relation to anaphylaxis and certain clinical problems Arch Internal Med 11 165
105 HIGGINS G M 1953 Hormonally induced eo inopenia of peritoneal fluid of white rats Am J Clin Path 23 775–783

106 HOLMGREN H & O WILANDER 1937 Beitrag zur Kenntnis der Chemie und Function der ehrlich hen Mastzellen Z mikroskop anat Forsch 42 242-278
107 HOMMA E 1921 Pathologische und biologische Untersuchungen über die eosinophil Zellen und die Eosinophilie Arch path Anat Physiol Virchow s 233 11
108 HUMPHREY J H & K SAQUES 1954 The histamine and serotonin content of the platelets and polymorphonuclear leucocytes of various species J Physiol 124 305-310
109 INGRAHAM F S & W B WARTMAN 1939 Chemotropism of human eosinophilic polymorphonuclear leukocytes Arch Path 28 318
110 JACKSON I J & B ROSE 1949 Observations on the histamine content of cerebrospinal fluid in man J Lab Clin Med 34 250-254
111 JOACHIM G 1906 Uber mastzellen Leukemien Deut Arch Klin Med 87 437
112 JOLLY J 1900 Clasmatocytes et Mastzellen Compt rend soc biol 52 609
113 JORPES J E 1946 Heparin 2nd ed Oxford Univ Press New York N Y
114 JORPES J J ODEBLAD & H BASTROM 1953 *An autoradiographic study on the uptake of S^35 labelled sodium sulphate in the mast cells* Acta haemat Base 9 273-276
115 JOSEY A I & J S LAWRENCE 1932 Studies in the physiology of the eosinophil II The effect of subcutaneous and intraperitoneal injections of *B coli* on the white blood cells of the guinea pig Folia Haematol 48 313-322
116 JOSEY A I & J S LAWRENCE 1932 Studies in the physiology of the eosinophil IV The effect of intraperitoneal and intravenous injection of sodium bicarbonate solution on the white blood cell picture Folia Haematol 48 330-336
117 JOSEY A I & J S LAWRENCE 1932 Studies in the physiology of the eosinophil III The effect of the intraperitoneal injection of ammonium chloride on the white blood cells of the guinea pig Folia Haematol 48 323 329
118 JOSEY A I 1933 Studies in physiology of eosinophil V Role of eosinophils in inflammation Folia Haematol 51 80-95
119 KAMEL A 1942 Is eosinophil leukocyte normally present in smears from conjunctiva? Bull Ophthalmol Soc Egypt 35 97-100
120 KATZ G 1940 Histamine release from blood cells in anaphylaxis *in vitro* Science 91 221
121 KATZ G & S COHEN 1941 Experimental evidence for histamine release in allergy J Am Med Assoc 117 1782-1783
122 KAUFMAN R E & F VOM SAAL 1940 Leucocyte changes in acute peritoneal irritation J Lab Clin Med 26 468-476
123 KIRK R C 1942 The causes of eosinophilia Intern Clin 1 219-233
124 KLINE B S M B COHEN & S A RUDOLPH 1932 Histologic changes in allergic and non allergic wheals J Allergy 3 531-541
125 KNOTT F Q & R G B PEARSON 1934 Eosinophilia in allergic conditions Guy s Hosp Repts 84 230
126 KOANG N K K I OU T I IVANG K L TCHEN & M I HSU 1951 Levels of circulating eosinophil and their use as an index of prognosis in surgical shock Chinese Med J 69 6-10 369-388
127 KOKSAL M 1953 Extraction of a heparin like substance from mast cell granules in mouse connective tissue Nature 172 733-734
128 KOVACS A 1950 Antihistamine effect of eosinophil leukocytes Experientia 6 349-350
129 KRAGH J & A HORGAARD 1943 Method of counting eosinophil cells in nasal secretion of patients suffering from allergic rhinitis Acta Oto-Laryngol 31 445-453
130 LARSH J E JR & J NICOLS 1949 Effect of adrenalectomy on eosinophil response of rats infected with *Trichinella spiralis* Proc Soc Exptl Biol Med 71 652-654
131 LARSSON F & A SWENSSON 1952 On the technique of eosinophil counting Scand J Clin & Clin Invest 4 162 164
132 LAST J H P JORDAN I PITESKY & F BOND 1950 The eosinophil response immediate vs delayed eosinopenia Science 112 47-49
133 LISON L 1935 Etudes sur la métachromasie colorants métachromatiques et substances chromatropés Arch biol Paris 46 599-668
134 LISON L 1935 La signification histochimique de la métachromasie Compt rend soc biol 118 821-824
135a LOFFLER W A F ESSELLIER G DEMEYER & I MORANDI 1952 Flüchtige lungen Infiltrate mit Bluteosinophilie nach therapeutischen Oliinjectionen Schweiz med Wochsch 83 771

135b MacIntosh F C & W D M Paton 1949 The liberation of histamine by certain organic bases J Physiol 109 190–219
136 MacMurray F G S Katz & H J Zimmerman 1950 Pleural fluid eosinophilia New Engl J Med 243 330–334
137 Marshall P B 1943 The influence of adrenal cortical deficiency on the histamine content of rat tissues J Physiol 102 180–190
138 Martin H & L Roka 1953 The heparin content of mast cells Acta Haematol 10 26–31
139 Mason M F 1939 Heparin A review of its history chemistry physiology and clinical application Surgery 5 451 618
140 Maximow A 1913 Untersuchen uber Blut und Bindegewebe VI Blutmastzellen Arch mickroskop Anat Entwicklungsmech 83 247
141 McIntire F C L W Roth & M Sproull 1950 *In vitro* histamine release from sensitized rabbit blood cells Evidence against participation of fibrinolysin Proc Soc Exptl Biol Med 73 605
142 Michels N A 1938 The mast cell Handbook of Hematology 1 237 H Downey Ed Hoeber New York N Y
143 Milchner R 1899 Uber die Emigration von mast Zellen bei myelogener Leukemie Z klin Med 37 194
144 Moore J E III & G W James III 1953 A simple direct method for absolute basophil leucocyte count Proc Soc Exptl Biol Med 82 601–603
145 Mora M D 1951 Valeur diagnostique et prognostique de l eosinophilie dans les maladies allergiques (Diagnostic and prognostic significance of eosinophilia in allergic disease) Acta allerg 4 253
146 Morelle L 1931 Sur le comportement des leucocytes basophiles dans certain chocs expérimentaux Compt rend soc biol 107 904
147 Moses C & M Flatt 1951 Changes in the total circulating eosinophil count in cyclotron workers Science 113 676
148 Muehrcke R C J L Lewis & F M Kark 1952 The effects of cortisone and heparin in eosinophils in defibrinated human blood Science 115 377–378
149 Murray R C 1953 The use of the absolute eosinophil count in the diagnosis of neoplasms preliminary report New Engl J Med 248 848–850
150 Nordmann M 1931 Local reactions in sensitized animals Physiol Revs 11 41–67
151 Nylander P E A A Pekkarinen L Aro & M Turunen 1953 Blood eosinophil count in the evaluation of indications for surgical operations on the lungs Ann chir gyn fenn 42 1–27
152 Oliver J F Bloom & C Mangieri 1947 On the origin of heparin J Exptl Med 86 107–116
153 Opie E 1903 The occurrence of cells with eosinophilic granulations and their relation to nutrition Am J Med Sci 117 217
154 Opie E 1904 The relation of cells with eosinophilic granulation to bacterial infection Am J Med Sci 127 988
155 Opie E 1904 An experimental study of the relation of cells with eosinophile granulation to infection with an animal parasite (*Trichinella spiralis*) Am J Med Sci 127 477
156 Opie E L 1929 Inflammation and immunity J Immunol 17 329–342
157 Padawer J & A S Gordon 1952 Eosinopenia and degenerating eosinophilic leukocytes in blood Proc Soc Exptl Biol Med 80 581–582
158 Padawer J & A S Gordon 1952 A mechanism for the eosinopenia induced by cortisone and by epinephrine Endocrinology 51 2 58
159 Paff G H & F Bloom 1949 Vacuolation and the release of heparin in mast cell cultivated *in vitro* Anat Record 104 45
160 Lanzenhagen H & K Speir 1953 Effect of horse serum adrenal hormones and histamine on the number of eosinophils in the blood and peritoneal fluid of mice Blood 8 536–544
161 Pfeifer A 1930 Eosinophilie und Allergie Deut Arch klin Med 169 65
162 Pilot M L 1950 Use of base in fluid for counting eosinophils a method for staining eosinophil Am J Clin Path 20 870 871
163 Prochek I & A Pappenheim 1910 Die experimentell Erzeugung von ... philen Exsudaten Fola Haemat 1 2 43
164 Quick A J 1944 The anticoagulants effective in vivo with special reference to heparin and dicumarol Physiol Revs 24 297–318
165 Randolph T G & F M Rackemann 1941 Blood histamine level in asthma and in eosinophilia J Allergy 12 450–456

166 RANDOLPH T G & C L STANTON 1945 Comparison of differential counts from stained film and counting chamber using propylene glycol aqueous stain Am J Clin Path Tech Sect 9 17 27

167 KREBCK J W 194 The functions of the white blood cells Am J Clin Path 17 614 623

168 RENOLD A E D JENKINS F H FORSHAM & G W THORN 1951 The use of intravenous ACTH A study of quantitative adrenocortical stimulation J Clin Endocrinol Metab 12 763)

169 RICH A R 1941 Significance of hypersensitivity in infections Physiol Revs 21 0–111

170 RILEY J F 1953 The effects of histamine liberators on the mast cells of the rat J Path Bact 65 4 1–4 9

171 RILEY J F 1953 Histamine in tissue mast cells Science 118 332

172 RILEY J F & G B WEST 19 3 The presence of histamine in tissue mast cells J Physiol 120 528 53

173 RINGOEN A 1923 The mast leukocytosis in adult guinea pigs under experimental conditions Am J Anat 31 319

174 RINGOEN A R 1938 Eosinophile leucocytes and eosinophilia Handbook of Hematology 6 179 H Downey Ed Hoeber New York N Y

175 ROCHA E & M SILVA 1947 The histamine theory of anaphylactic shock Arch Path 33 38 –408

176 ROCHA E & M SILVA 1944 Recent advances concerning the histamine problem J Allergy 15 399–413

•177 ROCHE M G THORN & A HILLS 1950 Levels of circulating eosinophils and their response to ACTH in surgery their use as index of adrenocortical function New Engl J Med 242 30 314

178 Reference cancelled

179 ROGER B O J M CONVERSE A C TAYLOR & R M CAMPBELL 1955 Eosinophile in human skin homografting Proc Soc Exptl Biol Med 82 523–526

180 ROMANI J D 1952 Essai d interprétation du phenomène de migration intratissulaire de polynucléaires éosinophiles au cours de la réaction d alarme Compt rend soc biol 146 1680

181 ROMANI J D 1953 L influence de la cortisone sur les mastocytes du tissu conjonctif et de quelques organes chez le rat Compt rend soc biol 147 391–394

182 ROSE B & J S L BROWNE 1941 The effect of adrenalectomy on the histamine content of the tissues of the rat Am J Physiol 131 589–594

183 ROSE B 1952 Histamine hormones and hypersensitivity Rec Prog Hor Res 7 375 398

184 ROSEMBERG E J CORNFIELD R W BATES & F ANDERSON 1954 Bioassay of adrenal steroids in blood and urine based on eosinophil response A statistical analysis Endocrinology 54 363–383

185 ROSEMBERG F & R A LEWIS 1950 Reduction in eosinophil level of adrenalectomized mice following injection of adrenocorticotropin 11-dehydro-17 hydroxycorticosterone and lipid extracts of human urine J Applied Physiol 3 164–172

186 ROUS F P 1908 Some differential counts of the cells in the lymph of the dog their bearing on problems in haematology J Exptl Med 10 537

187 RUD F 1947 The eosinophil count in health and in mental disease Acta psychiat et Neurol Suppl 40

188 SABRAZES J 1926 Leucémie à mastzellen et à cellules de ferrata rabiorés stantes Arch D Mal Du Coeur 19 453

189 SAKURADA H 1928 Differentiation of eosinophil and pseudo-eosinophil cells by means of peroxidase reaction (copper method) on smear and in counting chamber Tohoku J Exptl Med 11 1 5

190 SAMTER M 1949 The response of eosinophils in the guinea pig to sensitization anaphylaxis and various drugs J Hematol 4 217 246

191 SAMTER M M KOFOED & W PIEPER 1953 A factor in lungs of anaphylactically shocked guinea pigs which can induce eosinophilia in normal animals Blood 8 10 8 1090

192 SAMTER M F E ERICKSON & M A KOFOED 1952 A The effect of ACTH on the distribution of eosinophils in blood and peribronchial tissue of guinea pigs J Allergy 23 140–150

193 SCHLECHT H 1912 Über lokale Eosinophilie beim anaphylaktischen Versuch Verhandl Kong innere Med Wiesbaden 416

194 SCHLECHT H & G SCHWENKER 1912 Über die Beziehungen der Eosinophilie zur Anaphylaxie Deut Arch klin Med 108 405

135b MacIntosh F C & W D M Paton 1949 The liberation of histamine by certain organic bases J Physiol 109 190–219

136 MacMurray F G S Katz & H J Zimmerman 1950 Pleural fluid eosinophilia New Engl J Med 243 330–334

137 Marshall P B 1 43 The influence of adrenal cortical deficiency on the histamine content of rat tissues J Physiol 102 180–190

138 Martin H & L Roka 1953 The heparin content of mast cells Acta Haematol 10 26–31

139 Mason M F 1939 Heparin A review of its history chemistry physiology and clinical application Surgery 5 451 618

140 Maximow A 1913 Untersuchen uber Blut und Bindegewebe VI Blutmastzellen Arch mickroskop Anat Entwicklungsmech 83 247

141 McIntire F C I W Roth & M Sproull 1950 *In vitro* histamine release from sensitized rabbit blood cells Evidence against participation of fibrinolysin Proc Soc Exptl Biol Med 73 605

142 Michels N A 1938 The mast cell Handbook of Hematology 1 23/ H Downey Ed Hoeber New York N Y

143 Milchner R 1899 Uber die Emigration von mast Zellen bei myelogener Leukemie Z klin Med 37 194

144 Moore J E III & G W James III 1953 A simple direct method for absolute basophil leucocyte count Proc Soc Exptl Biol Med 82 601 603

145 Mora M D 1951 Valeur diagnostique et prognostique de l eosinophilie dans les maladies allergiques (Diagnostic and prognostic significance of eosinophilia in allergic diseases) Acta allerg 4 253

146 Morelle L 1931 Sur le comportement des leucocytes basophiles dans certain chocs expérimentaux Compt rend soc biol 107 904

147 Moses C & M Ilatt 1951 Changes in the total circulating eosinophil count in cyclotron workers Science 113 676

148 Muehrcke R C J L Lewis & R M Kark 1952 The effects of cortisone and heparin in eosinophils in defibrinated human blood Science 115 377–378

149 Murray R C 1953 The use of the absolute eosinophil count in the diagnosis of neoplasms preliminary report New Engl J Med 248 848–850

150 Nordmann M 1931 Local reactions in sensitized animals Physiol Revs 11 41–62

151 Nylander I E A A Iekkarinen L Aro & M Turunen 1953 Blood eosinophil count in the evaluation of indications for surgical operations on the lungs Ann chir gyn fenn 42 -22

152 Oliver J F Bloom & C Mangieri 1947 On the origin of heparin J Exptl Med 86 107–116

153 Opie E 1905 The occurrence of cells with eosinophilic granulations and their relation to nutrition Am J Med Sci 117 217

154 Opie E 1904 The relation of cells with eosinophilic granulation to bacterial infection Am J Med Sci 127 988

155 Opie E 1904 An experimental study of the relation of cell with eosinophile granulation to infection with an animal parasite (*Trichinella spiralis*) Am J Med Sci 127 477

156 Opie E L 1922 Inflammation and immunity J Immunol 17 329–342

157 Padawer J & A S Gordon 1952 Eosinopenia and degenerating eosinophilic leukocytes in blood Proc Soc Exptl Biol Med 80 581–582

158 Padawer J & A S Gordon 1952 A mechanism for the eosinopenia induced by cortisone and by epinephrine Endocrinology 51 52 8

159 Paff G H & F Bloom 1949 Vacuolation and the release of heparin in mast cell cultivated *in vitro* Anat Rec d 104 45

160 Panzenhagen H & P Speirs 1953 Effect of horse serum adrenal hormones and histamine on the number of eosinophils in the blood and peritoneal fluid of mice Blood 8 536–544

161 Peipers A 1930 Eosinophilie und Allergie Deut Arch klin Med 169 65

162 Pilot M I 1950 U of base in fluids for counting eosinophils a method for staining eosinophils Am J Clin Path 20 870 871

163 Frocher F & A Pappenheim 190 Uber experimentelle Erzeugung von e n philen Exsudaten Fola Haemat 1 2 543

164 Quick A J 1944 The anticoagulants effective *in vivo* with special reference to heparin and dicumarol Physiol Revs 24 297 318

165 Randolph T G & F M Rackemann 1941 Blood histamine level in asthma and in eosinophilia J Allergy 12 450–456

224 Vaughn J 1954 Eosinophils in the sputum Brit Med J No 4852 27–28
225 Vaughn J 1952 The stimulation of the eosinophil leucocyte J Path Bact 64 91 107
226 Vaughn J 1953 The function of the eosinophile leukocyte Blood 8 1 15
227 Velzeboer C M 1953 Eosinophil infiltration in the retina and choroid Ophthalmologica 126 187–188
228 Vercauteren R 1953 The properties of the isolated granules from blood eosinophiles Enzymologia 16 1 13
229 Vercauteren R & C Peeters 1952 On the presence of an antihistaminicum in isolated eosinophilic granulocytes Arch intern pharmacol therap 89 10–14
230 Waters E T S Markowitz & L B Jaques 1938 Anaphylaxis in the liverless dog and observations on the anticoagulant of anaphylactic shock Science 87 582 583
231 Webb R L 1931 Peritoneal reactions in the white rat with especial reference to the mast cells Am J Anat 49 283
232 Weidenreich F 1908 Morphologische und experimentelle Untersuchungen über Entstehung und Bedeutung der eosinophilen Leukocyten Verhandl Anat Ges Berlin 22 81
233 Weidenreich F 1910 Die Morphologie der Blutzellen und ihre Beziehungen zueinander Anat Record 4 317
234 Weidenreich F 190 Über die zelligen Elemente der Lymphe und der serosen Hohlen Verhandl Anat Ges Wurzburg 21 51
235 Weinberg M & P Seguin 1914 Recherches biologiques sur l'eosinophilie Ann inst Pasteur 28 470–508
236 Weld C B & J J Spitzer 1952 Hemolytic action of heparin in lipemic animals Am J Physiol 169 726
237 Wiener S 1952 The enumeration of eosinophil cells analysis of errors involved Med J Australia 1 633–635
238 Wilander O 1938 Studien über Heparin Skand Arch Physiol 81 Suppl 15
239 Wilson A 1941 The effect of adrenal ct my on the blood histamine of rabbits J Physiol 99 241 245
240 Wislocki G & E W Dempsey 1946 Observations on the chemical cytology of normal blood and hemopoietic tissues Anat Record 96 249 278
241 Wragg L L & K Speirs 19 ? Strain and sex differences in response of inbred mice to adrenal cortical hormones Proc Soc Exptl Biol Med 80 680–684
242 Wright A J W Baker W K Merrington & O Cope 1953 The elb and flux of the eosinophils in the burned patient and their use in the clinical management Ann Surg 137 175–183
243 Zappert J 1892 Eine Methode zur Zahlung der eosinophilen Zellen im frischen Blut Ze tr Klin Med 380
244 Zitcer E M W H Elsasser & P I Kirk 1953 Cultivation and morphology of human tissue mast cells *in vitro* Growth 17 111 172
245 Zollinger H U 1950 Gewebmastzellen und Heparin phasenmikroskopische Untersuchung (Tissue mast cells and heparin study with the phase microscope) Experientia 6 384–386

195 Schoch E P Jr & D Glick 1953 The effect of cold stress ACTH cortisone pyrogen and nitrogen mustard on tissue mast cells in the skin and subcutaneous tissues of the rat J Investigative Dermatol 20 119-132
196 Schoen I L Strauss & M Bay 1953 An evaluation of the eosinophil count in patients undergoing major surgery Surg Gynecol Obstet 96 403-408
197 Schwarz E 1914 Die Lehre von der allgemeinen und ortlichen Eosinophie Ergeb allg Path u path Anat 17 137 789
198 Selle W A 1946 Histamine—its physiological pharmacological and clinical significance Texas Repts Biol Med 4 138-244
199 Sevitt S 1951 Eosinophil and other leucocyte changes in burned patients Brit Med J 4713 976-995
200 Shoji K 1928 Differentiation of eosinophil and pseudo eosinophil leukocytes in rabbit blood by use of the oxidase reaction (Smear method and counting chamber method) Tôhoku J Exptl Med 11 6-9
201 Smith R W R R Margulis M J Brennan & R W Monto 1950 The influence of ACTH and cortisone on certain factors of blood coagulation Science 112 295
202 Snellman O B Sylven & C Julen 1951 Analysis of the native heparin lipoprotein complex including the identification of a heparin complement obtained from extracts of tissue mast cells Biochim et Biophys Acta 7 98 109
203 Speirs R S 1952 The principles of eosinophil diluents Blood 7 550-554
204 Speirs R S 1952 An improved eosinophil counting slide J Lab Clin Med 39 963-967
205 Speirs R S 1953 Assay of ACTH activity based on the eosinopenic response in normal and hypophysectomized mice Endocrinology 52 300-310
206 Speirs R S & R K Meyer 1949 The effects of stress adrenal and adrenocorticotrophic hormones on the circulating eosinophils of mice Endocrinology 45 403-429
207 Speirs R S & R K Meyer 1951 A method of assaying adrenal cortical hormones based on a decrease in the circulating eosinophil cells of adrenalectomized mice Endocrinology 48 316-326
208 Spitzer J J 1954 Comparison of the lipemia clearing and anticoagulant activities of heparin Am J Physiol 177 337 340
209 Sundberg R D F E Schaar M J S Powell & D Denboer 1954 Tissue mast cells in human umbilical cord and the anticoagulant activity of dried extracts of cords and placentae Anat Record 118 35
210 Swank R L & E S Roth 1954 Hemolysis and alimentary lipemia Effect of incubation heparin and protamine Blood 9 348-361
211 Swank R L A E Franklin & J H Quastel 19 0 Effects of fat meals and heparin on blood plasma composition as shown by paper chromatography Proc Soc Exptl Biol Med 75 850
212 Swank R L & V Wilmot 1951 Chylomicra their composition and their fate after intravenous injections of mall amounts of heparin Am J Physiol 167 403
213 Swanson J N W Bauer & M Ropes 1952 The evaluation of eosinophil counts Lancet 1 129-133
214 Taliaferro W H 1929 The Immunology of Parasitic Infections Century New York N Y
215 Tassi R 1950 Comparative observations on eosinophilia of the blood secretions and tissues in allergic nasal disorders Riv clin patol pharm 5 281-317
216 Taubert M 1951 Basophile Leukozyten und Heparin Z ges inn Med 6 758
217 Thayer W S 1897 On the increase of the eosinophilic cells in the circulating blood in trichinosis Lancet 2 78
218 Thorn G W 1953 The eosinophil ACTH epinephrine and stress Am J Med 14 139
219 Thorn G W & P H Forsham 1949 Metabolic changes in man following adrenal and pituitary hormone administration Rec Prog Hormone Res 4 229-288
220 Uhrbrand H 1953 The direct counting of circulating eosinophils Value of the method of ACTH and adrenaline tests Acta Haematol 9 121-134
221 Urbach K F & L Giscafre 1948 Identification of histamine from the white cell layer of centrifuged rabbit blood Proc Soc Exptl Biol Med 68 430-431
222 Valentine W N M L Pearce & J S Lawrence 1950 Studies on the histamine content of blood with special reference to leukemia leukemoid reactions and leukocytoses Blood 5 623-647
223 Vallery Radot P P Blamoutier F Claude & P Girold 1926 Eosinophilia in anaphylaxis Presse méd 34 1601 1604

scattered throughout the cytoplasm or aggregated into a rosette in the area of the centriole, the finer vacuoles lying toward the center of the rosette, depending on whether the cell is in motion or quiescent and rounded (FIGURES 4 to 6). The specificity of this rosette arrangement has been brought into controversy as regards lymphocytes on the one hand and plasma cells on the other hand.[4] It is true that both of the latter cells normally contain a few vacuoles stainable with neutral red and may contain considerable numbers of them under physiologic or pathologic stimulation. One needs to be wary of such stimulated forms in drawing deductions concerning intercellular relationships, and one must also turn to all other possible experimental approaches before drawing conclusions. But by and large neither lymphocytes nor plasma cells offer problems in the *differentiation of monocytes. The size of the monocyte, the abundance of its phagocytic vacuoles and the way it tends to arrange them, the abundance and smallness of its mitochondria, the pattern of its motility* and its differences from lymphocytes and plasma cells in these respects are distinctive and dependable.

The monocyte can, of course, be differentiated by fixed techniques for normal blood, but it rarely can be differentiated by those techniques from other mononuclear cells of pathologic blood or of the lymph and tissues. Doctor Sabin and her co-workers therefore naturally subjected the hematopoietic tissues to various types of investigation by this (then new) technique.

Under usual circumstances the *monocyte is not phagocytic of vital dyes* despite the ease with which it stains supravitally. The larger mononuclear phagocytes, on the other hand (i.e., the macrophages, the so-called resting wandering cells or histiocytes of connective tissues and the reticular and specific endothelial cells of the parenchymatous organs), stain both vitally and supravitally. It was on the basis of these facts and of certain embryologic studies that the pioneer workers with the monocyte came to the conclusion that it is a distinct cytologic entity unrelated to these larger phagocytes.[7] This conclusion met contradictions by the work of succeeding experimenters using either similar methods[14, 15, 35] or tissue cultures[17, 19] and the view now accepted by workers with all techniques seems to be that the morphology of the monocyte represents merely a specific physiologic status in the life of a cell that is normally destined to become a macrophage.[4, 7, 54] In other words, it is a young cell on its way to growing up, just beginning to be actively phagocytic and not yet capable of taking vital dyes, let alone taking the mass of tissue debris and foreign substances which is the function of the macrophage. The steps by which the monocyte acquires increasing phagocytic capacity and increasing enzymatic powers for digestion of the variety of substances handled by macrophages are yet to be revealed.

The cell was found to be a normal inhabitant, along with varying numbers of macrophages, of the lymphnodes,[14] spleen,[7, 4] bone marrow[14, 27] and loose connective tissues.[27] It was found to occur in large numbers about early tubercles, and it has since been shown to appear in abundant numbers in the first few days of inflammation of the connective tissues almost irrespective of the cause of the inflammation[15, 4, 42] (FIGURES 6 to 8). This influx of monocytes early in inflammation, and the fact that they are accompanied by other

THE MONOCYTE*

By Edna H Tompkins
Cancer Research Institute New England Deaconess Hospital Boston Mass

The monocyte owes its name and morphologic specificity to the supravital technique for the staining of blood and tissues By this technique is indicated the staining of living tissues after the death of the host in contradistinction to vital staining which indicates the staining of living tissues *in vita* or to fixed staining which indicates the staining of dead tissues Supravital staining is dependent upon the viability of the tissue and fades upon its death It is achieved only by certain types of dyes but discussion of this consideration is irrelevant here

I myself am an ardent supporter of the possibilities inherent in the supravital technique for the study of the physiology etiology and identity of hematopoietic tissues I believe that the possibilities combined with use of a gamut of unexplored specific stains and specific pretreatments of the tissues are tremendous Despite my enthusiasm for this technique I realize that some of the concepts in fundamental hematology which were formulated by the early workers with it have met sufficient revision over the years to bring them into accord with opposing concepts based upon other techniques and I suspect that other concepts of theirs which still remain controversial may have depended upon too sweeping an interpretation without sufficient appreciation of the importance of species site of biologic activity degree of biologic stress and possibly other unrecognized factors I believe that future work directed toward evaluation of these factors may serve to end the existing controversies

It is my hope in this discussion to present directions in which the concepts of those who pioneered in supravital studies seem to have been or seem likely to be modified and brought more in line with the concepts based upon other types of study The monocyte seems to stand at the crossroad as it were between the controversies involving the potentialities of lymphocytes and reticular cells on the one hand and of plasma cells and macrophages on the other and discussion of it touches inevitably upon all of these controversies

Simpson[30, 31] and Sabin[26] first demonstrated the definitiveness of the supravital morphology of the monocyte and showed that the cell comprises the two forms in the blood which had been considered separately up to that time namely the transitional and large mononuclear cells of Ehrlich s terminology The monocyte is motile and both it and its nucleus vary in shape depending on its movements It contains many fine rodlike mitochondria which stain with janus green or pinacyanol and which are scattered throughout the cytoplasm except in the area of the centriole (FIGURES 1 2 3) The most distinctive feature of the cell consists of a considerable number of vacuoles which concentrate the supravital dye neutral red These vacuoles stain according to the neutral reaction of the dye and vary in size They may be

* Th w k po t d th p pe w d [illegible] U t d St t At m E gy C mm [illegible] AT(30 1) 901 w th th N w E gl d De[illegible] Hosp t l



All illustrations represent photographs of living tissues and cells which were stained supravitally with neutral red and viewed at 37° C. unless otherwise indicated. Vacuoles stained with the dye appear black in the photographs. All photographs were taken with ordinary bright field illumination and all magnifications are × 2070 except where indicated.

[illegible] A monocyte [illegible] The blood [illegible] × 3347


1


2



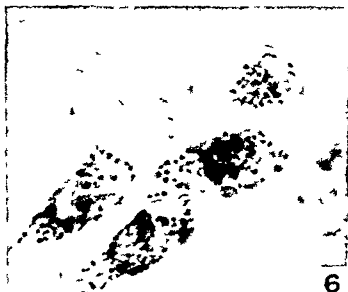
All illustrations represent photographs of living tissues and cells which were stained supravitally with neutral red and viewed at 37 C unless otherwise indicated Vacuoles stained with the dye appear black in the photographs All photographs were taken with ordinary bright field illumination and all magnifications are X 20 0 except where indicated

F ure 1 2 13 M m blood Phase t tm F Am ocyt t h w th h act f th m t h 1 1 1 1 th h ge po t f ll d l d t am bo d m m t Th blood w un ta d d l ft t oom t mpe t Th ph t g ph w t k t t l f 10 m t betw t f d 2 d f tw m t betw 2 13 Th ll h d m d ts po t th fi ld be tw th fi t tw ph t g ph b t m ly t po t bn t p t betw th la t t ph t g ph L f t hl w th th l t h g ppe red po se t th locom tio f th cell N l l h w d t tly th m q sr t l 2 13 M toch d pe fi wh t od d ts th gl t th d p l m Th ph gocyt cu l ppe sc tt d da k bod M d um b ght ph se t t X 3347



FIGURE 4 M ocyte i h m blood The cell a r d d nd qu sc nt, a d the v c le of ne tr l d w rr ged a nd th c nt i l n cha ct r t r sette Th s ll wo ld be cla f d a large mononu l ll f th blood in fix d p pa at s

FIGURE 5 Sam f lm for FIGURE 4 A m nocyte wh h w lowly m g nd i wh ch th phag cyt cuoles w e th ef re s att red w tho t pe f a angem t Th cell wo ld be la s fied as a t a t al ll f the blood n fi d p p r t s



F URE 6 Mouse \ 4 Film f ubc ta ne t t s f om r th th d be i j t l l y ast nucl i d three day p ly The t w i t zed by a fil at f m ocyt w h w r small r d y ge th those f n rm l blo d Th d t f t l l w le ab d t l f smaller ze th those f blood mo ocyt s They w e l osett sc tt d th ugh t th y l plasm d pe d g on wh ther th c ll w s r t g nd l d m t o



F ure 7 A th film f om th same re f ure 6 H th y g m ocyt w re ll d d d se ral xh b ted h t t f gm tat on f th pe ph l cyt plasm w th t los f b l ty Th f gm t t h w p rt cula ly well th ll t th w

leukocytes from the blood and are at first small and less phagocytic and far more abundant than the monocytes of the blood stream offer difficulties as to the etiology of monocytes

The etiology of the monocyte represents in fact a long-existent controversy By means of depletions of the bone marrow of all definitive free cells Cunningham Sabin and Doan[7] demonstrated development of new cells of the granulocytic series direct from undifferentiated reticular cells by way of a free but still undifferentiated round cell which they called the primitive cell The primitive cell could not be differentiated from other basophilic progenitors in fixed tissues Cunningham *et al* found further that this cell also gives rise to both lymphocytes and monocytes each separately within the nodes and spleen and that it also occurs together with undifferentiated reticular cells in normal connective tissues and can provide local origin of monocytes upon stimulation Transitional forms between lymphocytes and monocytes were not obtained The authors therefore concluded that both cells are definitive and unrelated except through their common progenitor

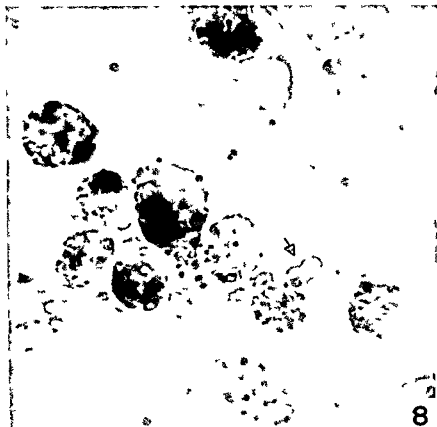


FIGURE 8 M 9 F lm f b t t t f m a th t had be j t d f d y f ly w th yea t l i Th ph t g ph l d ll th t d g f my g m cyt to m ph g P pl l f gm t t f m o yt without l f b l ty h w th ll t th r w

Investigators working on fixed tissues[3, 10, 18, 2, 3, 25] on the other hand particularly those who approached the subject by way of embryology or of inflammation within the connective tissues found what they considered to represent a continuous series of transitions between lymphocytes and macrophages and these transitions were regarded as also inclusive of monocytes These workers therefore concluded that monocytes develop directly from lymphocytes in the process of becoming macrophages The impossibility of differentiation of monocytes especially at early ages from other basophilic mononuclear cells in fixed preparations from organs and inflammatory reactions coupled with the demonstration of their development from free primitive cells in living preparations from tissues under different experimental duress, obviously presents the need for new approaches before the answer between these two concepts of origin can be certain

The controversy involves not only questions of the etiology but also questions concerning the functions of both lymphocytes and monocytes of the relationship of these cells to plasma cells and of the potentialities for cyto

genesis outside of the hematopoietic organs Numerous questions concerned with overproductions and metaplasias obviously hinge upon it In the mean time the possibility should be faced that both concepts are correct, depending upon location and biologic urgency in much the same way that macrophages arise largely by maturation of monocytes in the connective tissues but may arise directly from the undifferentiated reticular cell in parenchymatous organs or as the result of severe biologic depletions and thus by pass the stages of both primitive cell and monocyte It is interesting by the way, that this compromise represents essentially the concept of the origin of monocytes upheld by Downey and Weidenreich[10]

Irrespective of its origin the developed monocyte exhibits biologic activities which suggest that it is probably of greater potentiality than is appreciated It is obviously a young cell It is capable of prolific reproduction much of which is amitotic[7, 9] (FIGURE 9) Normally it builds the way to becoming the most powerful phagocyte of the body the macrophage[1, 34] (FIGURE 10) It undoubtedly therefore has potent capacities for growth for the production of enzyme systems and for development of the powers of phagocytosis and digestion The difficulty of differentiating the cell in the tissues and at younger stages than normally occur in the blood limits application of the newer histochemical techniques toward elucidation of its capacities It is obvious that the cell must contain at its more mature and phagocytic stages those enzymes that have been shown present in macrophages[34] but the ages at which these enzymes develop are uncertain As was said the cytoplasm contains abundant mitochondria at the age at which monocytes normally occur in the blood (FIGURES 1 2 3) and it therefore contains presumably most of the oxidative enzymes that are usually associated with mitochondria and ATP[30] It has been found to contain oxidases[34] peroxidases[39] acid and basic phosphatase[21] with several substrates including nucleic acid[41] lipids including phospholipid[8] and ribonucleo protein[41] The cell is obviously capable of protein synthesis at this stage Its capacity for phagocytosis suggests the likelihood that it can ingest numerous biologic colloids and digest or synthesize them accordingly It is interesting that the number of mitochrondria decreases as the cell matures to become a macrophage The relationship of this development to the type and quantity of enzymes at the latter stage is not known The monocyte is elicited by and phagocytic of phospholipids and is able to metabolize these lipids[27, 34] The cell was shown by Sabin and her co-workers to be elicited by lipoid fractions from tubercle bacilli[9] and by injections of a dye protein[9] and to exhibit shedding of its cytoplasm at a time when antibodies to the protein were multiplying in the blood stream It was therefore suggested that monocytes and macrophages represent the cytologic site for production of antibodies and that shedding is the mechanism by which they are delivered to the blood and tissue juices The support given by others to plasma cells[1, 2, 1] or lymphocytes[39, 40] as the site of this production emphasizes the need for elucidation of the relationships between these cells

Cytoplasmic shedding seems to represent a normal potentiality of young monocytes whatever may be the nature of the materials liberated thereby We have found such shedding within two to four days of local injections of

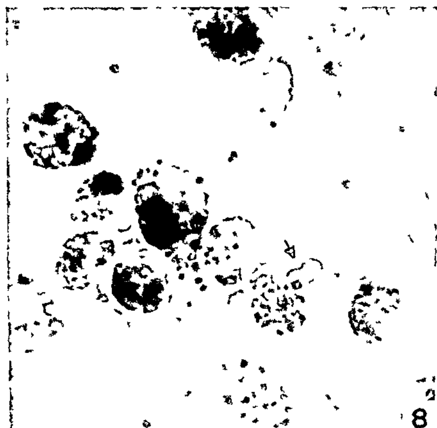
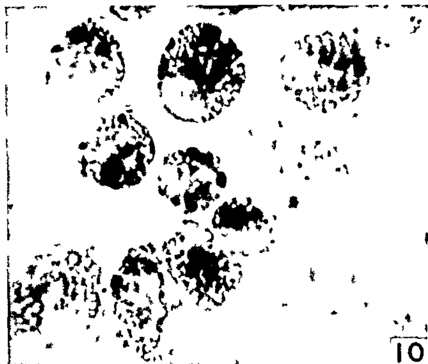


Figure 8 [illegible]

Investigators working on fixed tissues[3, 10, 13, 23, 25] on the other hand particularly those who approached the subject by way of embryology or of inflammation within the connective tissues found what they considered to represent a continuous series of transitions between lymphocytes and macrophages and these transitions were regarded as also inclusive of monocytes These workers therefore concluded that monocytes develop directly from lymphocytes in the process of becoming macrophages The impossibility of differentiation of monocytes especially at early ages from other basophilic mononuclear cells in fixed preparations from organs and inflammatory reactions coupled with the demonstration of their development from free primitive cells in living preparations from tissues under different experimental duress obviously presents the need for new approaches before the answer between these two concepts of origin can be certain

The controversy involves not only questions of the etiology but also questions concerning the functions of both lymphocytes and monocytes of the relationship of these cells to plasma cells and of the potentialities for cyto-



F t 10 Mouse \ ? F lm f a bcut ne u ne t t ss f om th t had be j ted w th y t le d tw day pre ou ly Th re w filt t d w th phagocytic ll t ll levels f t ty f um th t f y ung m ocyt t tha f m ph ge Th cell ta d d pl t f f t w ll f eutr l red P rph l f gm tat w p e t m y f th y ger lls

Cytoplasmic shedding is not the only method by which the cell seems able to give to the environment products of its own metabolic activities The vacuolar apparatus suggests another such mechanism The character of the vacuoles varies markedly according to that of the material ingested and may at times even be used as a biologic test for the presence of such materials We have found the vacuoles that stain with neutral red to be quite distinctive following injections of the phosphatide sphingomyelin [34] for instance or of cholesterol [35] These vacuoles are very distinctive in tuberculosis [4] Unknown as yet is the basic character of the material in the phagocytic vacuoles that enables them alone to stain with the neutral red while the major portion of the cytoplasm remains unstained Whatever this material is it is capable of a certain degree of modification depending on the materials ingested by the cells and there is evidence that it may be extruded from the vacuoles to the exterior from time to time in much the same way as with the contractile vacuoles of amoebas In the monocytes of normal rabbits in particular the vacuoles often assume pear shapes intermittently with long slender stems of dye leading to the surface (FIGURES 11–17) or the transformations in the vacuolar apparatus of macrophages in connective tissue after injections of



FIGURE 9 [illegible] day [illegible] phagocyt[illegible] [illegible] ×2300

nucleic acids (FIGURES 7 8) or of cholesterol[15] There was no other evidence supravitally of loss of viability of the monocytes involved Sabin[2] made similar observations in conducting her experiments with dye protein Contrary to her experience however we have rarely observed shedding at the later periods in our experiments when the reactions are characterized by more mature and phagocytic cells

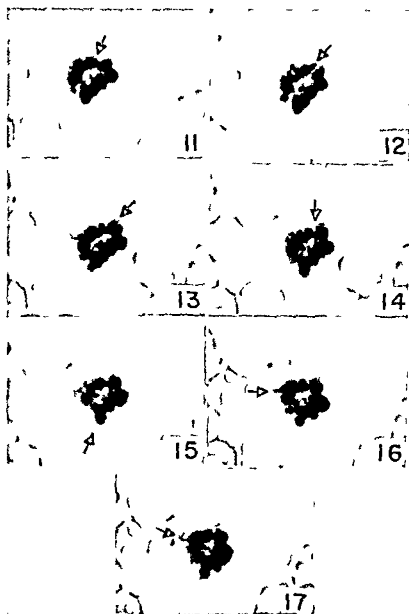
cholesterol may be offered as another illustration [35] These transformations can be followed as the vacuoles change from their normal color and size becoming dark and very small and ultimately disappearing Vacuoles of the normal type then reappear

Experimental evidence from my laboratory supports the probability that still another method functions to supply the organism with substances obtained from monocytes in moments of sufficient stress Dougherty and White[9] demonstrated that lymphopenia was followed by lymphocytosis at specific time intervals after parenteral injections of adrenal cortical extract They demonstrated a widespread fragmentation of lymphocytes and signs of irritation of the reticular cells at periods corresponding to the onset of lymphopenia[9] We have shown that monocytopenia also develops at the same interval of time following injection of adrenal cortical extract and is succeeded by a monocytosis[F] These findings are interpreted as presumptive evidence that disruption of monocytes as well as of lymphocytes follows upon admission of adrenal cortical extract to the body fluids and that the materials built into the bodies of monocytes must therefore also be added to the tissue juices

So much for the likely functions of the monocyte in its normal status *i e* multiplication phagocytosis analysis synthesis elaboration of the systems necessary for development into macrophages and liberation of certain of its products by cytoplasmic budding vacuolar extrusion and probably by fragmentation under adrenal stimulation The details of these activities need clarification

Modifications of the monocyte on the other hand occur so characteristically in specific pathologic states that they can serve not only as valuable diagnostic aids but also as indicators of the underlying activities of which monocytes are capable and which can become inhibited or seriously modified under those pathologic conditions Demonstration of such modifications has depended largely upon supravital studies since the modifications are apparently not evident in fixed tissues until they have become so drastic as to lead to cytologic death These modifications have been described in detail elsewhere[33] and will therefore be reviewed only to the extent necessary to round out this survey of the monocyte The changes in monocytes in the presence of tubercle bacilli have been shown by Sabin and her co-workers[*3] to be due specifically to the content of tuberculo thioic acid in the waxy coat of the bacillus The nuclei divide amitotically but the monocytes seem incapable of cytoplasmic division under the effects of that acid with the result that binucleated and finally multinucleated cells are formed The vacuolar apparatus becomes changed at the same time to dark red in color and to a state of uniform fineness and of accumulation about the centriole These are the characteristics typical of the epithelioid cell and by them individual epithelioid cells may be recognized without awaiting tubercle formation Intracellular fatty degeneration develops and the monocytes eventually die with liberation of the myriads of bacteria which have multiplied within during the processes It is not known how the tuberculo-thioic acid acts to cause these changes

Similar changes can be caused within both monocytes and macrophages by a wide variety of unrelated substances[15 28 32 4] Epitheloid cells also found



FIGURES 11 [illegible] Am[illegible]ocyt[illegible] blood Th[illegible] ph[illegible] w[illegible] tak[illegible] sequ[illegible] two [illegible] h w th [illegible] h pe[illegible] po[illegible] f th[illegible] ta[illegible] d w th [illegible] take pla[illegible] tantly [illegible] abl[illegible] Th[illegible] at th[illegible] ow fr[illegible] 11 w[illegible] [illegible] 12 [illegible] 13 [illegible] tra[illegible] 14 [illegible] them[illegible] re[illegible] sett[illegible] 14 to 17 [illegible] sett[illegible] bel[illegible] w the [illegible] sett[illegible] th sam[illegible] figu[illegible]e

19 Maximow A 1928 Arch exptl Zellforsch Gewebezücht 5 169
20 Palade G E 1952 Anat Record 114 427
21 Rabinovitch M & D Andreucci 1949 Blood 4 580
22 Rebuck J W 1940 Anat Record (Suppl) 78 46
23 Rebuck J W 1941 Am J Clin Path 16 614
24 Rebuck J W 1952 Federation Proc 11 474
25 Renaut J 1907 Arch d Anat microscop Par 9 495
26 Sabin F R 1923 Bull Johns Hopkins Hosp 34 277
27 Sabin F R C A Doan & R S Cunningham 1925 Contribs Embryol 82 125
28 Sabin F R 1932 Physiol Revs 12 141
29 Sabin F R 1939 J Exptl Med 70 67
30 Simpson M E 1921 Univ Calif (Berkeley) Pubs Anat 1 1
31 Simpson M E 1922 Anat Record 23 37
32 Tompkins E H 1936 Am J Syphilis Gonorrhea Venereal Diseases 20 22
33 Tompkins E H & R S Cunningham 1938 *In* Handbook of Hematology Hoeber New York N Y
34 Tompkins E H 1942 Bull Johns Hopkins Hosp 70 55
35 Tompkins E H 1946 Arch Path 42 239
36 Tompkins E H 1949 J Am Med Womens Assoc 4 447
37 Tompkins E H 1952 J Lab Clin Med 39 365
38 Washburn A H 1929 J Lab Clin Med 14 246
39 Wesslén T 1952 Acta Dermato-Venereol 32 265
40 White A & T F Dougherty 1946 Ann N Y Acad Sci 48 859
41 Wislocki G B & E W Dempsey 1946 Anat Record 96 249
42 Wright A W 1930 Am J Path 6 8

loosely scattered all through the lymphomata of Hodgkin s disease,[33] serve to differentiate the disease from purely infectious involvements of the nodes or from other forms of malignant lymphomata

The vacuolar apparatus of monocytes also becomes fine in anemia and malaria but the vacuoles are sparse and scattered instead of occurring in rosette formation The vacuoles are brownish in hue and the cells evince no abnormalities of division or evidences of degeneration

In contrast to these conditions in which the phagocytic vacuoles become fine and attenuated the vacuoles become coarse and abundant under a number of circumstances [33] This occurs in ordinary pyogenic infections in which large drops of fat also accumulate in the cells It is also the case in infections caused by *Bacillus milletensis* and *Bacillus monocytogenes* in which the monocytes also multiply and actually become more like macrophages The vacuoles also become greatly enlarged in the lipoid storage diseases [33]

These instances all illustrate conditions in which monocytes respond specifically but in a controlled manner to specific stimuli Monocytes may, however exhibit the uncontrolled multiplications which characterize tumor growth and leukemia [11] [33] The multiplication may occur at levels of extreme youth in which case the cells are but slightly phagocytic and exhibit characteristics of the reticular cell The multiplication may occur at stages of phagocytic activity which are more characteristic of normal monocytes, or it may occur at stages of phagocytosis active enough for the cells to be considered macrophages It is to this last stage that Lawrence applied the term clasmatocytic leukemia [16] In all cases the clinical picture is one of acute leukemia As in other malignant lymphomata and leukemias understanding of the etiology of monocytes and of the stimuli which lead to their production and multiplication or to their maturation into macrophages with the development of increased phagocytic and digestive powers will be necessary before the forces which lead to uncontrolled multiplication can be elucidated

References

1 Bing J A Fagraeus & B Thorell 1945 Acta Physiol Scand 10 282
2 Bjørneboe M & H Gormsen 1943 Acta Path Microbiol Scand 20 649
3 Bloom W 1928 Folia Hematol 37 1
4 Bloom W 1928 Folia Hematol 37 63
5 Bloom M L & G B Wislocki 1950 Blood 5 79
6 Cunningham R S F R Sabin S Sugiyama & J A Kindwall 1925 Bull Johns Hopkins Hosp 37 231
7 Cunningham R S F R Sabin & C A Doan 1925 Bull Johns Hopkins Hosp 84 227
8 Dougherty T F & A White 1944 *Endocrinology* 35 1
9 Dougherty T F & A White 1945 Am J Anat 77 81
10 Downey H & F Weidenreich 1912 Arch mikroskop Anat Entwicklungsmech 80 306
11 Downey H 1938 Handbook of Hematology Hoeber New York N Y
12 Ehrich W F D L Drabkin & C Forman 1949 J Exptl Med 90 157
13 Epstein R D & F H Tompkins 1943 Am J Med Sci 206 249
14 Forkner C E 1930 J Exptl Med 52 385
15 Gray M E 1940 Am J Anat 67 361
16 Lawrence J S A I Joley & M W Young 1931 Folia Hematol 44 332
17 Lewis M 1925 Am J Path 1 91
18 Maximow A 1923 Arch mikroskop Anat Entwicklungsmech 97 623

Plate I Kindred 1938a) *Each secondary nodule is roughly divided into two* zones a light staining zone (L) next to the marginal sinus and a dark zone (D) at the medullary end In Peyer s patches the dark zones of the secondary *nodules abut against the tunica muscularis of the intestinal wall and the light* zone with its crowning mantle zone projects into the mucosa In the spleen there is no differentiation into light and dark zones Such topographic arrangements of the zones as are present in the secondary nodules of the lymph nodes of the rat are also characteristic of those in man dog and cat These data supported the view of Kohlich (1933) as to the differentiation of the secondary nodules into dark and light zones

These qualitative observations were followed by quantitative estimates of *the cell populations and relative mitotic indices of the lymphocytes and retic* ulum cells in the several regions of the lymph nodes and other lymphoid organs including the spleen and thymus For the sake of brevity and in illustration of the methods used these quantitative results have been gathered together and compared with similar observations on these organs in young rats (Kindred 1938b and c 1939 1940) and in young adult rats (1942 1947) The measurements of the 1947 group were the controls for the study of the effects of the nitrogen mustards on the hemopoietic organs of rats The statistical *methods of Fisher (1934) for contrast of variations in small quantities was used* throughout and the variance designated in the table is the standard error of the mean

In TABLE 1 are shown the quantitative characteristics of the cortex of the cervical lymph nodes (this includes the mantle zones of the nodules and the *internodular pulp*) It can be seen that the numbers of cells per unit volume (100 000 cu mμ) are about the same throughout the stages studied The incidences of the small lymphocytes are the same The populations of the *medium sized and large lymphocytes which are the germinal cells of this tissue* fluctuate in each age and have the greatest incidence at 30 days and the least at 60 to 80 days Reticulum cells appear to have about the same incidence

TABLE 1

CERVICAL LYMPH NODES CORTEX OUTSIDE OF NODULES CELLS PER 100 000 CU Mμ PERCENTAGE DISTRIBUTIONS OF CELLS PER UNIT VOLUME AND CELLS IN MITOSIS

Date	1940	1940	1938b	1938a 1942	194
Days	15	20	30	60–80	60–80
Cells per unit volume	300	300	309 ± 5	348 ± 14	386 ± 18
Small lymphocytes	91 4	94 0	6 0 ± 2 8	94 0 ± 0 3	97 0 ± 2 0
Medium lymphocytes	3	2	20 0 ± 2 6	1 4 ± 0 2	1 8 ± 0 3
Large lymphocytes	2 1	1	1 5 ± 0 3	0 5 ± 0 1	0 5 ± 0 06
Reticulum cells	2 3	2 4	2 4 ± 0 4	4 4 ± 1 2	4 0 ± 0 5
Macrophages	neg	neg	neg	neg	0 05 ± 0 04
Degenerate lymphocytes	neg	neg	neg	neg	1 2 ± 0 8
Mitosis					
Lymphocytes	1 6	1 3	1 3	4 7 ± 1 2	3 ± 0 6
Reticulum cells	0 5	0 9	0 8	0 3 ± 0 04	0 5 ± 0 08

QUANTITATIVE STUDIES ON LYMPHOID TISSUES

By James E Kindred

Department of Anatomy Medical School University of Virginia Charlottesville Va

The quantitative studies covered in this paper started as an inquiry into the structure of the secondary lymphoid nodule (Kindred 1936, 1937 and 1938a) At about the same time Jordan (1939) as a result of his own studies and those with Speidel had sought to relate his observations on the origin and fate of the lymphocyte to the quantitative observations of Yoffey (1933 and 1936) and others before him, that more lymphocytes enter the blood stream daily via the thoracic duct than are present in the blood stream at any one time More evidence for this view was presented by Yoffey and Drinker (1939), but no quantitative objective analyses as to the origin of these lymphocytes had been made These investigators stated that while it is generally accepted that lymphocytes are formed in the lymph nodes tonsils Peyer s patches and solitary nodules and that they enter the lymph stream from these organs and also enter the circulation via the blood capillaries of the spleen, there was no general acceptance that the thymus was a lymphoid organ

It was under these circumstances that the quantitative methods presented here in brief were started I wished to begin my study with observations on the histology of the lymphoid tissue of the submaxillary lymph nodes of the rat but found that there were conflicting views as to the function of the secondary nodules These views current in 1935 were in brief as follows (1) Flemming's original view (1885) that the secondary nodule is a center for the production of lymphocytes (2) Hellman s view (1921) that the secondary nodule is a center of reaction against noxious products brought into the nodule through the lymph stream and (3) Rohlich s view (1933) that the secondary nodule is a germinal center for the production of lymphocytes and also a center of reaction to substances brought into the nodules through the blood stream

In the first investigation I sought to make a quantitative investigation of the cellular elements and of such morphologic characteristics as are indicative of function in order to test the validity of these conclusions Albino rats were used as a basis for the observations but the lymphoid organs of man dog and cat were used for comparative purposes In the rat sections were made from submaxillary (anterior cervical) inguinal and mesenteric lymph nodes and Peyer s patches and from the spleen of 80 day old litter mates (Kindred 1936 1937 and 1938a) Rats from the colony of Doctor Alfred Chanutin of the Department of Biochemistry of the University of Virginia were used in this study and in all subsequent investigations of rat hemopoietic organs All of the material was fixed in Helly s fluid and stained either with H & E or eosin azure II and hematoxylin

From the qualitative survey of this material it was established that the secondary nodules of the lymph nodes are prolate spheroids or spheres lying subjacent to the marginal sinus The outer end of the nodule is capped by a cup-shaped layer of small lymphocytes the mantle zone (M in the figure of

TABLE 3

CERVICAL LYMPH NODES SECONDARY NODULES DARK ZONES CELLS ETC

Date	1938	1938a–1947	194
Days	30	60 80	60 80
Cells per unit volume	285 ± 3	250 ± 13	225 ± 27
Small lymphocytes	26 0 ± 1 8	13 ± 2 0	21 0 ± 5 0
Medium lymphocytes	60 0 ± 1 8	8 6 ± 2 0	56 7 ± 3 0
Large lymphocytes	8 0 ± 0 4	2 8 ± 0 3	4 1 ± 0 6
Reticulum cells	3 2 ± 0 3	2 4 ± 0 2	2 ± 0 3
Macrophages	3 2 ± 0 2	3 2 ± 0 3	3 1 ± 0 2
Degenerate lymphocytes	—	5/per macrophage	13 4 ± 1 1
Mitosis			
Lymphocytes	3 5 ± 0 5	3 4 ± 0 6	4 0 ± 1 0
Reticulum cells	3 ± 1 8	1 1 ± 0 2	1 8 ± 0 2

in the percentage distributions because the degenerative effects of nitrogen mustard poisoning were being measured The mitotic indices of the lymphocytes remain the same throughout but those of the reticulum cells are less in the older rats

The significant differences between the light and dark zones of the secondary nodules lie in the higher incidences of medium sized lymphocytes and higher mitotic index of the lymphocytes in the dark zone also in the relatively smaller populations of small lymphocytes reticulum cells and macrophages in the dark zones From these data (Kindred 1938) it was concluded that the quantitative evidence supports the view of Rohlich (1933) that there are two different regions in the lymphoid nodules only as regards the mitotic index of lymphocytes and that phagocytosis by the macrophages is about the same in both regions These conclusions have been supported by observations on the lymph nodes from other animals and from other regions of the rat (Kindred 1938a and 1947)

The relative percentage incidences of the lymphocytes plasma cells and reticulum cells together with the mitotic indices of the lymphocytes and reticulum cells of the medullary cords of the cervical nodes are shown in TABLE 4 The small lymphocytes have smaller relative incidences in the older rats than in the younger while medium sized lymphocytes and plasma cells have greater incidences It would appear that small lymphocytes as they age are transformed into plasma cells and medium sized lymphocytes and are not replaced Large lymphocytes do not bulk large in the population Reticulum cells have significantly smaller incidence in the older stages and may possibly account for some of the lymphocytes and plasma cells Macrophages and cell destruction are generally negligible in the cords in the early stages and are at a minimum compared with other parts of the nodes In the early stages the mitotic indices are high and in the older rats low The cell production is apparently able to take care of the wear and tear of local conditions but does not supply cells for use in other parts of the body Russell body cells and young and old granulocytes form varying proportions of the population of the medullary cords

TABLE 2

CERVICAL LYMPH NODES CELLS PER 100 000 CU Mμ PERCENTAGE DISTRIBUTION OF CELLS AND CELLS IN MITOSIS IN LIGHT ZONES OF SECONDARY NODULES

Date	1938b	1938a–1942
Days	30	60 80
Cells per unit volume	230 ± 15	240 ± 13
Small lymphocytes	29 0 ± 2 2	48 0 ± 4 9
Medium lymphocytes	60 0 ± 1 4	40 0 ± 4 5
Large lymphocytes	7 0 ± 1 1	3 3 ± 0 4
Reticulum cells	2 3 ± 0 3	4 0 ± 0 3
Macrophages	3 0 ± 0 4	4 7 ± 0 5
Mitosis		
Lymphocytes	1 7 ± 0 3	1 9 ± 0 9
Reticulum cells	8 3 ± 1 2	0 8 ± 0 2

throughout although there is a trend toward increase in the older stages Macrophages were not observed in this region of the cortex in the youngest rats and in the older rats they appear as modifications of the reticulum cells in response to damage to the lymphocytes (Kindred, 1947) The mitotic indices of the lymphocytes appear to increase in the older rats whereas those of the reticulum cells are constant These data are the constants for the distributions of cells and their mitosis rates which have been used in analyzing the production of cells from one age to the next in 15 day old and 20 day old rats (Kindred 1940) and for lymphocyte production from this region of lymph nodes in older rats (Kindred 1942 and 1947)

In TABLE 2 are shown the distributions of cells in the light (Rohlich s phagocytic) zones of the nodules of the cervical lymph nodes Nodules do not start to develop until after the 20 day stage so none is included in rats earlier than 30 days The number of cells per unit volume is considerably less than in the mantle zones and there is no significant difference with age The small lymphocytes have greater incidence at 60 days than at 30 and the medium sized lymphocytes and large lymphocytes have relatively less but still make up a significant part of the population Reticulum cells and macrophages have about the same incidence in the nodules of the older as of the younger rats The mitotic indices of the lymphocytes are about the same in both stages but the mitotic index of the reticulum cells is significantly less in the older rats

Similar quantitative data on the cells of the dark (Rohlich s mitotic) zones of the secondary nodules of the cervical lymph nodes are shown in TABLE 3 In 30 day-old rats when the nodules are just beginning to form the number of cells per unit volume is much more variable than it is in the later stages All of the cells fluctuate in number Small lymphocytes are less numerous and medium sized lymphocytes are more numerous in this part of the nodule than they are in the light zone Large lymphocytes decrease significantly with age Degenerated lymphocytes (inside and outside of macrophages) not present in great numbers in the nodules of the young rats are numerous in the nodules of the older rats and in the latest counts (1947) were included

TABLE 3

CERVICAL LYMPH NODES SECONDARY NODULE DARK ZONES CELLS ETC.

Date	1938	1938a 1942	[illegible]
Days	30	60 80	60 80
Cells per unit volume	285 ± 37	250 ± 13	225 ± 2[illegible]
Small lymphocytes	26.0 ± 1.8	13.7 ± 2.0	21.0 ± 5.0
Medium lymphocytes	60.0 ± 1.8	78.6 ± 2.0	56.[illegible] ± 3.0
Large lymphocytes	8.0 ± 0.4	2.8 ± 0.3	4.1 ± 0.6
Reticulum cells	3.2 ± 0.3	2.4 ± 0.2	[illegible] ± 0.3
Macrophages	3.2 ± 0.2	3.[illegible] ± 0.3	3.1 ± 0.2
Degenerate lymphocytes	—	5/per macrophage	13.4 ± 1.1
Mitosis			
Lymphocytes	3.5 ± 0.5	3.4 ± 0.6	4.0 ± 1.0
Reticulum cells	3 ± 1.8	1.1 ± 0.2	1.8 ± 0.2

in the percentage distributions because the degenerative effects of nitrogen mustard poisoning were being measured. The mitotic indices of the lymphocytes remain the same throughout but those of the reticulum cells are less in the older rats.

The significant differences between the light and dark zones of the secondary nodules lie in the higher incidences of medium sized lymphocytes and higher mitotic index of the lymphocytes in the dark zone also in the relatively smaller populations of small lymphocytes reticulum cells and macrophages in the dark zones. From these data (Kindred 1938) it was concluded that the quantitative evidence supports the view of Rohlich (1933) that there are two different regions in the lymphoid nodules only as regard the mitotic index of lymphocytes and that phagocytosis by the macrophages is about the same in both regions. These conclusions have been supported by observations on the lymph nodes from other animals and from other regions of the rat (Kindred 1938a and 1942).

The relative percentage incidences of the lymphocytes plasma cells and reticulum cells together with the mitotic indices of the lymphocytes and reticulum cells of the medullary cord of the cervical nodes are shown in TABLE 4. The small lymphocytes have smaller relative incidences in the older rats than in the younger while medium sized lymphocytes and plasma cell have greater incidences. It would appear that small lymphocytes as they age are transformed into plasma cells and medium sized lymphocytes and are not replaced. Large lymphocytes do not bulk large in the population. Reticulum cells have significantly smaller incidence in the older stages and may possibly account for some of the lymphocytes and plasma cells. Macrophages and cell destruction are generally negligible in the cords in the early stages and are at a minimum compared with other parts of the nodes. In the early stages the mitotic indices are high and in the older rats low. The cell production is apparently able to take care of the wear and tear of local conditions but does not supply cells for use in other parts of the body. Russell body cell and young and old granulocytes form varying proportions of the population of the medullary cords.

TABLE 4

CERVICAL LYMPH NODES MEDULLARY CORDS CELLS ETC

Date	1940	1940	1938b	1938a–1947
Days	15	20	30	60–80
Cells per unit volume	—	—	—	—
Small lymphocytes	36 5	45 0	22 0 ± 1 7	6 9 ± 0 7
Medium lymphocytes	11 0	16 0	24 0 ± 1 5	41 0 ± 4 0
Large lymphocytes	1 8	9 2	7 4 ± 2 1	7 3 ± 1 0
Plasma cells	9 0	12 0	40 5 ± 3 4	40 6 ± 4 3
Reticulum cell	11 5	10 0	4 0 ± 0 2	4 5 ± 0 4
Macrophages	neg	neg	neg	0 9 ± 0 07
Mitosis				
Lymphocytes	6 5	1 2	3 3	0 6 ± 0 06
Reticulum cell	1 4	0 7	1 5	0 2 ± 0 07

R ss ll b dy lls d gr locytes f rm ry g pr p rt f th pop l t

TABLE 5

THYMUS CORTEX CELLS ETC

Date	1940	1940	1938b	1938a–1947	1947
Days	15	20	30	60–80	60–80
Cells per unit volume	390	390	330 ± 15	412 ± 8	470 ± 16
Small lymphocytes	58 0	59 0	68 0 ± 3	79 0 ± 1 5	90 0 ± 1 7
Medium lymphocytes	28 0	28 0	27 0 ± 2 4	18 0 ± 1 5	5 2 ± 0 09
Large lymphocytes	2 2	1 8	1 0 ± 0 1	0 8 ± 0 05	0 5 ± 0 004
Reticulum cells	*11 0*	*11 2*	*3 2 ± 0 1*	*2 4 ± 0 3*	*2 3 ± 0 3*
Macrophages	—	—	—	0 1 ± 0 07	neg
Degenerate lymphocytes	—	—	—	1 6/per macrophage	2 7 ± 0 6
Mitosis					
Lymphocytes	15 0	15 0	16 0 ± 4 0	1 4 ± 0 2	7 0 ± 3 0
Reticulum cells	4 1	5 8	1 0 ± 0 1	0 7 ± 0 1	0 7 ± 0 4

The thymus has been found to be the most important lymphocyte producing organ in young rats (Kindred 1940) It is considered to account not only for its own growth but to contribute lymphocytes necessary for the growth of other lymphoid organs and for the needs of lymphocytes by the circulating blood (Kindred 1940) Any damage to its cells is reflected in an acute lymphopenia (Kindred 1947) The comparative data on the quantitative histology of the thymic cortex are shown in TABLE 5 In the early stages the lymphocyte population is fluctuating and there is a high incidence of medium sized and large lymphocytes These lymphocytes have high mitotic indices and in some 60 to 80-day old rats a relatively high mitotic rate is maintained Small lymphocytes are produced in great numbers in the cortex and the size of the cells decreases with age Reticulum cells are quite numerous in the early stages and are also mitotically active producing reticulum cells and possibly some of the lymphocytes In contrast to the nodules of the lymph nodes there is no high phagocytic rate and macrophages do not appear until the lymphocytes are poisoned by some such agent as the nitrogen mustards

TABLE 6
SPLEEN LYMPHOID CORDS CELLS, ETC

Date	1940	1940	1938, 1942	194[illegible]
Days	15	20	60–80	60–80
Cells per unit volume	50	280	[illegible]0 ± [illegible]	263 ± 13
Small lymphocytes	81.[illegible]	85.[illegible]	91.5 ± 0.8	8[illegible].0 ± 1.4
Medium lymphocytes	8.[illegible]	7.7	1.8 ± 0.3	3.[illegible] ± 0.6
Large lymphocytes	0	1.3	0.3 ± 0.2	0.6 ± 0.1
Reticulum cells	10.0	5.8	5.[illegible] ± 0.6	5.5 ± 0.[illegible]
Macrophages	neg	neg	neg	0.5 ± 0.2
Degenerate lymphocytes	—	—		[illegible].7 ± 0.8
Mitosis				
Lymphocytes	1.8 ± 1.3	1.[illegible]	[illegible].9 ± 0.3	1.8 ± 0.3
Reticulum cells	1.1 ± 1	0.8	0.3 ± 0.1	–

(Kindred 194[illegible]) Such a condition in the normal thymus suggests that this region is only a source of lymphocytes while the secondary nodules of the lymph nodes and, as we shall see, of the spleen are not only sites of proliferation but are regions where effete lymphocytes are eliminated and the byproducts from nucleic acid destruction are utilized in the rejuvenation of the resident lymphocytes, a process somewhat resembling the manner in which Walker (1954) has suggested that products resulting from enucleation of the normoblasts are utilized by the erythroblasts in erythropoiesis. That the nucleic acid persists in the macrophages for some time is shown by the positive reaction of the dead nuclei to Feulgen's reagent. No one has as yet shown that there is such a turnover of nucleic acid but Andreassen and Ottensen (1944) have shown that in P32 marked rats the thymus is the site of greatest P32 turnover in the series of lymphoid organs examined. As such this site is followed by the mesenteric lymph nodes, skin nodes, spleen and Peyer's patches.

In TABLE 6 the quantitative characteristics of the splenic cords are summarized. The number of cells per unit volume remains about the same throughout the ages studied. Small lymphocytes increase with age while medium sized lymphocytes decrease. Large lymphocytes remain about the same. Reticulum cells decrease and macrophages are negligible. A few degenerated lymphocytes are present in the older stages. No appreciable number of other types of cells was found in the lymphoid cords. The mitotic indices are low and remain low throughout.

Although the cortex of the lymph nodes, the cortex of the thymus and the splenic cords are regarded as dense lymphoid tissue, there are certain quantitative differences by which they can be distinguished, such as (1) the more constant and high percentage of small lymphocytes in the lymph nodes, (2) the greater incidence of medium sized lymphocytes in the thymic cortex, and (3) the higher incidence of reticulum cells in the splenic cord. Also in the early stages mitosis is never so high in the lymph node cortex nor in the splenic cords as it is in the thymic cortex.

The secondary nodules of the spleen have no light zones. The quantitative histologic characteristics of these nodules are summarized in TABLE 7. The

TABLE 7

SPLEEN SECONDARY NODULES CELLS ETC

Date	1938b	1938a–1942	1947
Days	30	60–80	60 80
Cells per unit volume	—	193 ± 28	216 ± 5.0
Small lymphocytes	72.0	28.0 ± 4.3	24.0 ± 5.0
Medium lymphocytes	14.0	56.0 ± 3.0	57.0 ± 5.0
Large lymphocytes	3.6	6.8 ± 1.2	3.0 ± 0.3
Reticulum cells	9.6	5.8 ± 0.[illegible]	3.0 ± 0.8
Macrophages	0.8	3.3 ± 0.6	2.5 ± 0.1
Degenerate lymphocytes	—	—	11.3 ± 1.4
Mitosis			
Lymphocytes	0.[illegible]	4.8 ± 1.0	2.3 ± 0.3
Reticulum cells	0.2	0.4 ± 0.2	—

population is extremely variable In the nodules of the 30 day old rats the small lymphocytes predominate but in the older rats there is a greater population of medium sized lymphocytes Large lymphocytes are quite noticeable even though not abundant Reticulum cells decrease with age and macrophages are few in the nodules of the younger stage A few secondary nodules are present before the 30 day old stage Degenerated lymphocytes are not marked until the 60 day old stage The mitotic indices of the lymphocytes increase with age but those of the reticulum cells remain low

In contrast with the secondary nodules of the cervical lymph nodes those of the spleen resemble more the dark zones than the light zones and yet there are certain differences from both zones particularly as regards small lymphocytes and reticulum cells in the nodules of the early stage Both of these types of *cells have higher incidences in the splenic nodules than in those of the cervical* nodes Medium sized lymphocytes and degenerated lymphocytes and the mitotic index of the lymphocytes are about the same as in the dark zones of the nodules

The data on the 15 day old and 20 day-old rats together with additional similar quantitative data on other hemopoietic organs were used in a formula from the calculus devised by Doctor J F McShane of the Mathematics Department of the University of Virginia to calculate hourly production of lymphocytes and other cells on the basis of varied lengths of time for the duration (one to three hours) of the mitotic cycles (Kindred 1940)

The data on the 60- to 80 day old rats supplemented by additional data on the lymphoid organs and bone marrow were utilized to predict the production of different kinds of cells needed to meet the demands of growth and of cells for the circulating blood The calculations were based on mitotic cycles with a length of either 30 minutes or one hour Two interesting features were introduced into the methods of study In the first place the area covered by the epithelium of the small intestine and enumeration of the lymphocytes in this epithelium were calculated for evidences of migration and loss via the lumen Although the calculated number of small lymphocytes found in the epithelium and the subjacent lamina propria mucosae was 125,000,000

± 32 000 000/100 gm body weight it was concluded that there was no evidence that the e cells were on their way out becaus• in the intact epithelium the intercellular cement of the terminal bars demonstrated as present in the intestinal epithelium by Chambers and Renyi (1925) was presumed to act as a bar to emigration Damage to the epithelium such as occurs in radiation and nitrogen mustard poi oning was believed to permit large numbers of lymphocytes to escape along this route (Kindred 1947)

The second feature of lymphocyte life not emphasized in the earlier studies was the estimation of the loss of small lymphocytes by phagocytosis in the secondary nodules The number destroyed per hour was estimated by multiplying the average number of small lymphocytes engulfed by the macrophages by the macrophage population and dividing this number by 24 hours the number of hours which has been recorded by Clark and Clark (1930) as the time required for the nucleus of an ingested nucleated cell to disappear following ingestion by a macrophage in the extravascular tissue of the tail of the living frog tadpole Thus an estimate of mall lymphocyte destruction was included in the calculations concerning lymphocyte production in response to the needs of growth in both local ituations and in the circulating blood (Kindred 1942)

Calculations made on the relations of the lymphatic drainage (Job 1915) to the production of newly formed lymphocytes showed that about 50 per cent of these would be contained in lymphatics which drained into the thoracic duct 37 per cent by the right lymphatic channels and 13 per cent via the blood vessels of the spleen The total number of lymphocytes entering the blood stream via the thoracic duct per hour/100 gm body weight would be about 8 2 per cent of the estimated number of lymphocytes in the circulating blood When Yoffey s (1933) data from measurements of the number of lymphocytes entering the blood via the thoracic duct in the dog were analyzed in the same way it was found that the lymphocytes amount to about 5 0 per cent of the number of lymphocytes in the circulating blood/100 gm body weight This agreement between calculated production and measurements of lymphocytes entering the blood gives quantitative support to the view of Yoffey and Drinker (1939) that the vast majority of lymphocytes entering the blood through the lymph stream were newly formed in the lymphoid organs

These quantitative methods of study were used in analyzing the effects of the nitrogen mustards on the hemopoietic organs of rats from the same colony (Kindred 1947) and of dogs (Kindred 1949) and were found to be very successful in presenting quantitatively the changes in the hemopoietic organs associated with leucopenia

In an analysis of the life span of the white blood cells as measured in irradiated parabiotic rats Van Dyke and Huff (1951) have found that my calculations for the production of myelocytes and lymphocytes are in remarkable agreement with the findings reported in their paper

References

ANDREASSEN F & J OTTESEN 1944 Significance of the various lymph id organs to the lymphocyte production in the albino rat Acta Path Microb ol Scand Suppl 54 25–32

CHAMBERS R & G RENYI 1925 The structure of the cells in tissues as revealed by microdissection I The physical relationships of the cells in the epithelia Am J Anat 35 385–403

CLARK, E I & E L CLARK 1930 Observations on macrophages of living amphibian larvae Am J Anat 46 91–147

FISHER R A 1934 Statistical Methods for Research Workers Oliver & Boyd London England

FLEMMING A 1885 Studien über die Regeneration der Gewebe Arch mikroskop Anat Entwicklungsmech 24 50–91

HELLMAN T J 1921 Studien über das lymphoide Gewebe Die Bedeutung der Sekundarfollikel Beitr path Anat u allgem Path 68 333–363

JOB T T 1915 The adult anatomy of the lymphatic system in the common rat (*Epimys norvegicus*) Anat Record 9 447–458

JORDAN, H E 1939 The lymphocytes in relation to erythrocyte production Anat Record 73 227–241

KINDRED J E 1936 An interpretation of the secondary lymphoid nodule in lymph nodes of the albino rat Anat Record 64(Suppl 3) 27

KINDRED J E 1937 An interpretation of the secondary lymphoid nodules in the albino rat Coll Net 12 110–111

KINDRED J E 1938a A quantitative study of the lymphoid organs of the albino rat Am J Anat 62 453–473

KINDRED J E 1938b A quantitative histologic study of the lymphoid organs of 20-day and 30 day old rats Anat Record 70(Suppl 3) 45

KINDRED J E 1938c A quantitative study of the histology of the lymph nodes of the submaxillary region of 20-day old albino rats Anat Record 72(Suppl) 76

KINDRED J E 1939 A quantitative study of the hemopoietic organs of young albino rats Biol Bull 77 314

KINDRED J E 1940 A quantitative study of the hemopoietic organs of young albino rats Am J Anat 67 99–149

KINDRED J E 1942 A quantitative study of the hemopoietic organs of young adult albino rats Am J Anat 71 207–243

KINDRED J E 1947 Histologic changes occurring in the hemopoietic organs of albino rats after single injections of 2 chloroethyl vesicants Arch Path 43 253 295

KINDRED J E 1949 The blood cells and the hemopoietic and other organs of dogs given intravenous injections of 2 chloroethyl vesicants Arch Path 47 378 398

RÖHLICH K 1933 Struktur und Blutgefässversorgung der Keimzentren Anat Anz 76 215–222

VAN DYKE D C & R L HUFF 1951 Life span of white blood cells as measured in irradiated parabiotic rats Am J Physiol 165 341 347

WIECKER H 1954 Exakte Kriterien der Knochenmarks die Masz und Mengenrelationen der Erythroblasten als Ausdruck der Leifungs und Teilungsgesetze der Erythropoese Schweiz med Wochschr 84 245 264

YOFFEY J M 1933 The quantitative study of lymphocyte production J Anat 67 250–262

YOFFEY J M 1936 Variation in lymphocyte production J Anat 70 507–514

YOFFEY J M & G K DRINKER 1939 The cell content of peripheral lymph and its bearing on the problem of the circulation of the lymphocyte Anat Record 73 417–427

Discussion of the Paper

DOCTOR WILLIAM BLOOM (*University of Chicago, Chicago Ill*) After graduation from medical school I spent two years in pathology At night and on week ends when the more experienced pathologists were not present I had to try to make diagnoses on blood smears and I often found myself in great difficulty The pretty pictures in the books often did not look like the cells in my smears and I was frequently unable to verify some of the supposed minute differences in cells

When I began to work with Doctor Maximow one of the first questions I asked him was how could one tell a lymphoblast from a myeloblast At that time I knew nothing of what he stood for in hematology He looked at me

sharply grunted and said I have been trying to do that for over 20 years without success I cannot express how relieved I was when I heard him make this remark To this day I cannot tell these cells apart

As I worked in the field I learned how many criteria had been proposed from time to time in attempts to solve the problem of the morphological separation of the free stem cells of the blood into several categories Incidentally I believe that much of the acrimony in this field was in part a result of the Franco-Prussian War Thus most of the Germans valiantly defending the position taken by Ehrlich and later by Naegeli were for years unwilling even to consider seriously the findings and theories of French investigators and vice versa

I might mention some of the more important of the differentiating criteria which have fallen by the wayside For instance it was claimed for some years that one could tell the blasts apart by the number of their nucleoli there being supposedly one nucleolus in lymphoblasts and many in myeloblasts but after a few years this supposition was generally admitted not to be true The matter of nucleoli in blood cells was carried to extremes when it was even claimed that some types of cells did not possess nucleoli This assertion of course was an error introduced by the dry smear method since all of the cells contain nucleoli Then it was maintained that one could tell the blasts apart by their mitochondria (the Altmann Schridde granules) but this claim too was soon found to be invalid Next it was said that one could tell these cells apart by a positive oxidase reaction in myeloblasts and a negative one in lymphoblasts But this criterion was also deficient because all of the supposedly different stem cells are oxidase negative it being essentially the specifically granulated cells which give the oxidase reaction (possibly excepting weak oxidase reactions in some monocytes)

Then came a revival of supravital staining using Janus green and neutral red In my experience this method does not enable one to tell the various stem cells apart As I told my former teacher Doctor Sabin it seemed to me after a thorough trial of the supravital method that one made a diagnosis essentially on the organ in which the stem cells are found calling them myeloblasts in bone marrow monoblasts in spleen and lymphoblasts in lymph nodes The diagnosis of a cell type was accordingly made not on distinguishing morphological characteristics but on the types of accompanying cells and I found the method worthless in trying to analyze the stem cells in extramedullary myelopoiesis and in circulating normal and pathologic blood I would also point out that the neutral red rosette is not specific for monocytes In our rats over 50 per cent of the circulating lymphocytes contained neutral red rosettes and of course these cells are not monocytes There is a lymph node in the rat between the cecum and ileum which is loaded with plasma cells and these cells contain rosettes I am sure our rats with possible exception of Bartonella infection were healthy animals Again as I recall Hall found that a very high percentage of the circulating lymphocytes in the monkey also had rosettes

I shall not consider other minor distinguishing criteria which were proposed

CHAMBERS R & G RENYI 1925 The structure of the cells in tissues as revealed by microdissection I The physical relationships of the cells in the epithelia Am J Anat 35 385–407

CLARK E R & E L CLARK 1930 Observations on macrophages of living amphibian larvae Am J Anat 46 91–147

FISHER R A 1934 Statistical Methods for Research Workers Oliver & Boyd London England

FLEMMING A 1885 Studien uber die Regeneration der Gewebe Arch mikroskop Anat Entwicklungsmech 24 50–91

HELLMAN T J 1921 Studien uber das lymphoide Gewebe Die Bedeutung der Secondarfollikel Beitr path Anat u allgem Path 68 333–363

JOB T T 1915 The adult anatomy of the lymphatic system in the common rat (*Epimys norvegicus*) Anat Record 9 447–458

JORDAN H E 1939 The lymphocytes in relation to erythrocyte production Anat Record 73 227–241

KINDRED J E 1936 An interpretation of the secondary lymphoid nodule in lymph nodes of the albino rat Anat Record 64(Suppl 3) 27

KINDRED J E 1937 An interpretation of the secondary lymphoid nodules in the albino rat Coll Net 12 110–111

KINDRED J E 1938a A quantitative study of the lymphoid organs of the albino rat Am J Anat 62 453–473

KINDRED J E 1938b A quantitative histologic study of the lymphoid organs of 20-day and 30 day old rats Anat Record 70(Suppl 3) 45

KINDRED J E 1938c A quantitative study of the histology of the lymph nodes of the submaxillary region of 20-day old albino rats Anat Record 72(Suppl) 46

KINDRED J E 1939 A quantitative study of the hemopoietic organs of young albino rats Biol Bull 77 314

KINDRED J E 1940 A quantitative study of the hemopoietic organs of young albino rats Am J Anat 67 99–149

KINDRED J E 1942 A quantitative study of the hemopoietic organs of young adult albino rats Am J Anat 71 207–243

KINDRED J E 1947 Histologic changes occurring in the hemopoietic organs of albino rats after single injections of 2-chloroethyl vesicants Arch Path 43 253 295

KINDRED J E 1949 The blood cells and the hemopoietic and other organs of dog given intravenous injections of 2 chloroethyl vesicants Arch Path 47 378 393

ROHLICH K 1933 Struktur und Blutgefässversorgung der Keimzentren Anat Anz 76 215–232

VAN DYKE D C & R L HUFF 1951 Life span of white blood cells as measured in irradiated parabiotic rats Am J Physiol 165 341–347

WIECKER H 1954 Exakte Kriterien der Knochenmarks die Masz und Men enrelationen der Erythroblasten als Ausdruck der Reifungs und Teilungsgesetze der Erythropoese Schweiz med Wochschr 84 245–264

YOFFEY J M 1933 The quantitative study of lymphocyte production J Anat 67 250–262

YOFFEY J M 1936 Variation in lymphocyte production J Anat 70 507–514

YOFFEY J M & C K DRINKER 1939 The cell content of peripheral lymph and its bearing on the problem of the circulation of the lymphocyte Anat Record 73 417–427

Discussion of the Paper

DOCTOR WILLIAM BLOOM (*University of Chicago Chicago Ill*) After graduation from medical school I spent two years in pathology At night and on week ends when the more experienced pathologists were not present I had to try to make diagnoses on blood smears and I often found myself in great difficulty The pretty pictures in the books often did not look like the cells in my smears and I was frequently unable to verify some of the supposed minute differences in cells

When I began to work with Doctor Maximow one of the first questions I asked him was how could one tell a lymphoblast from a myeloblast At that time I knew nothing of what he stood for in hematology He looked at me

Part II Techniques in the Study of Leukocytic Functions

A METHOD OF STUDYING LEUKOCYTIC FUNCTIONS *IN VIVO**

By John W Rebuck and James H Crowley†

The Department of Laboratories The Henry Ford Hospital Detroit Mich and The Department of Anatomy The University of Minnesota Minneapolis Minn

Introduction

Passage of the white cells of the blood from the blood vessels into the area of inflammation probably was first described by Dutrochet[1] in 1828 (Hektoen and Kiesman[2] Adami[3]) In 1843 Addison[4] immersed a frog s foot in hot water and noted that the colorless corpuscles in the irritated web of its foot adhered to the tissue and that the corpuscles went on congregating in the irritated tissue for an hour or two In 1846 Waller[5] also described the active nature of leukocytic migration Addison[6] moreover in 1849 anticipated Cohnheim s[7] more publicized experiment

Zimmerman[8] (1852) (*cf* Schlumberger[9]) emphasized that inflammation was a local process characterized by an abnormal escape of blood corpuscles from the vessels associated with a focal rise in temperature Virchow[10] (1858) could not decide whether leukocytes were pus cells taken into the blood or conversely if pus cells were extravasated leukocytes

Schultze[11] (1865) was able to observe white blood cells kept at body temperature by means of his ingeniously contrived warm stage In his Fig 13 he depicts a finely granular cell in the ameboid form now thought to be characteristic of a lymphocyte This cell had ingested fine droplets of milk which he had mixed with the human blood cells

Haeckel[12] (1862) injected indigo into the mollusc *Tethys* and was the first to demonstrate that leukocytes were capable of taking up foreign bodies into their own cytoplasm after finding indigo granules within the blood corpuscles

Migration of leukocytes from vessels was further confirmed by F E Schultze[13] (1866) Hering[14] (1867) and by Cohnheim[7] in his now famous paper of the same year

Lieberkuhn[15] (1870) demonstrated the ameboid motility of leukocytes in salamanders rabbits and various other animal species watching their advancement in small glass tubes He coined the phrase ameboid motion

Bizzozero[16] (1871 1872) observed leukocytes in the larger cells of pus and suspected that the larger cells had devoured the leukocytes Ziegler[17, 18] (1875 1876) was perhaps the first to advance the concept that mononuclear exudative cells were transformed mononuclear white blood cells corpuscles but unfortunately he soon abandoned the idea

According to Gay[1] Hayem Birsch Hirschfeld and Koch had previously demonstrated bacteria within the cells of diseased tissues but the true cyto

* W ... d D ... H ... D ... y f ... l ... h ... h ... w ... k

† J m ... H ... t ... l y ... se ... t ... H ... St J seph Hospital, St P ... l M

at one time or another but are now generally recognized as having no value But I must discuss the latest morphological criterion proposed for separating the blasts in which it was claimed that lymphocytes move with a 'hand mirror configuration with a little tail of cytoplasm trailing behind them and that the myeloblasts move with a corkscrew motion When I read this report I was sure that it could not be true because I had watched many of these cells in cultures over many years I started to repeat these observations with time lapse motion pictures, but this was just before the war and I became busy with other work Doctor De Bruyn took over the problem and did a lovely job with it He found that when the free stem cells irrespective of their organ of origin, were on the cover slip, they moved with a hand mirror motion and that when they were deep in the fibrin meshwork of the culture they moved with a corkscrew motion I can still recall the look on Doctor Wintrobe s face when we showed him motion pictures of a stem cell moving on the cover slip with a hand mirror form (supposed lymphocyte motion), then leaving the cover slip and crawling in the fibrin with corkscrew motion (supposed myelo blast motion) and assuming the supposed type of lymphocyte motion when it came back onto the cover slip again

So this method and all the other morphological methods proposed in the last half century singly and in combination have failed to enable me to dis tinguish the various blasts from one another even on morphological grounds The growing tendency of more and more clinical hematologists to speak of the stem cells as blasts until other discriminating criteria appear either in the blood cells or in the clinical pathological course of the diseases is a reflection of their inability to separate these supposedly different cells by the present mor phological methods In short we are not delimiting cells by their morphology if we have to know what their progeny will be before we can pigeonhole them

We need better and newer methods Surely the continued repetition of old claims based on the methods of the past half century have not helped us advance All that I have said so far is on the negative side

I cannot now go into a consideration of the great numbers of experiments on the histogenesis of inflammation extramedullary myelopoiesis transplantation of hematopoietic organs and tissue culture nor can I go into any details of normal embryogenetic and comparative hematopoiesis In my opinion this great body of observations substantiates the general thesis that for embryonic and adult animals including mammals the free stem cells in all blood cell forming tissues are morphologically and potentially identical in all situations and that this cell (lymphocyte hemoblast hemocytoblast or whatever other term is one s favorite name for it) develops in a particular direction according to the stimuli to which it is exposed

process needs no modern revision (his p 491) 'The (lymphocytes) nuclei are surrounded by a small amount of protoplasm of a pale rose color More careful examination of the lymphoid tissue reveals the presence of other mononucleated lymphocytes differing only slightly at first from the other cells surrounding them The nucleus of these cells is somewhat paler and its reticulum slightly more apparent though the protoplasm may not be at all increased in size as yet Other cells possess a nucleus which is again slightly larger paler and more vesicular than the latter as if the nuclear juice had increased at the expense of the solid part of the nucleus Lastly the nucleus and protoplasm become enormous and the macrophage is formed The macrophage is merely a lymphocyte which has assumed a new shape and a new function Excellent figures are appended to this paper

Metchnikoff's lectures[23] on the comparative pathology of inflammation delivered at the Pasteur Institute and published in 1892 demonstrated irrefutably the role of the lymphocyte as it hypertrophied to form first large mononuclears and then hematogenous macrophages

Arnold[28] (1893) introduced various foreign bodies into the mesentery and lymph sac of frogs and followed the gradual transitions of lymph cells to the large connective tissue elements in the ensuing aseptic inflammation Borrel[9] (1894) made similar observations in tubercles in the kidney of the rabbit Kanthack and Hardy[30] (1894–95) traced the origin of their hyaline cells (large mononuclear cells) to the lymphocytes of coelomic fluid and established them as morphological units distinct from the specifically granulated cells These investigators observed the same lymphocytic function in blister fluid from the skin of the arm of human volunteers into which *Bacillus ramosus* had been subsequently inoculated

Ranvier[31] (1900) studied the functions of clasmatocytes and leukocytes in aseptic inflammation of the peritoneum mesentery and appendix in the green frog salamander white rat guinea pig and rabbit He amply demonstrated that lymphocytes were a source of the defense elements

Maximow[32] (1902) in an extensive review of his experiments in aseptic inflammation described (1) the motility of lymphocytes (2) migration of lymphocytes from the smaller vessels into the field of inflammation and (3) their progressive hypertrophy into lymphocytogenous macrophages (his polyblasts) Maximow inserted a series of celloidin chambers into the connective tissues of rabbits dogs and pigeons More than 20 timed stages from 19 hours to 65 days of inflammation were so studied In 1903 the same investigator[33] extended his studies on new tissue growth in similar chambers from the fifth hour to the eight month in the process of scar formation He demonstrated that macrophages originated from newly emigrated lymphocytes and sessile clasmatocytes

In 1904 Maximow[34] while studying inflammatory connective tissue formation and changes in mast and fat cells of white rats found that in as short a period as four hours the hypertrophy of emigrated lymphocytes resulted in macrophage formation In the same year Ziegler[35] studied edema in the skin and subcutaneous tissues of 21 human subjects at autopsy He traced the gradual transformation of lymphocytes migrated from the blood vessels into

logical basis of natural resistance was established by the work of Metchnikoff. Although Metchnikoff gave full credit to the earlier work of other men (Panum, Crawitz, Caule and Roser) still, as expressed by Cay,[19] the full exposition and understanding of phagocytosis was Metchnikoff's own.

Through his work on mesodermic digestion in Medusae, Metchnikoff became convinced that mesoderm retained a primitive digestive function. Although the Coelentera and some Echinoderms possessed an entodermal digestive cavity, he believed that their mesodermal cells retained their powers of intracellular digestion. In 1883 he was struck by the idea that the mobile cells of a transparent starfish larva might serve as defense elements against invaders, whereupon he introduced rosethorns under the skin of a starfish larva (*Bipinnaria asterigera*). As a result the rosethorns were surrounded by the mobile mesodermal elements.[20, 21]

Soon afterward Metchnikoff observed spontaneous ingestion and destruction of *Monospora bicuspidata* by mobile cells of the blood of the infested fresh water crustacean *Daphnia magna* which had made their way into the general body cavity (his figures 41–43, 1891). Inflammation became to Metchnikoff in its simplest expression a reaction of mesodermic cells to an agent extrinsic to an organism. To these mesodermic cells which digested microbes he gave the name suggested to him by Claus, phagocytes, that is, devouring cells.

The following years saw the application of Metchnikoff's experiments[22, 23] to more complex animal forms with bacteria serving as stimuli to inflammation. In these forms the mesodermal elements acting as phagocytes were of two general types, macrophages and microphages. The microphages were the polymorphonuclear granulocytes of the blood and the macrophages were again subdivided into (1) hematogenous macrophages, that is, lymphocytes which in turn gave rise to the large mononuclears, and (2) histogenous macrophages, the phagocytic cells of the spleen, peritoneum, bone marrow, sinusoidal lining cells of the liver, and in general the fixed tissue cells now known as the *reticuloendothelial system of cells*.

Leber[24] (1888) first described and demonstrated chemotaxis of leukocytes. He watched their reaction to an extract of *Staphylococcus aureus* which had been placed in the anterior chambers of the eyes of his experimental animals.

The foundation for our modern concept of lymphocyte function is found in Metchnikoff's paper of 1888. In these experiments Metchnikoff demonstrated that lymphocytes of the blood of the rabbit and marmot suffering from pulmonary tuberculosis migrated into tuberculous areas and gradually hypertrophied to form cells which he called large mononuclears. These large mononuclears in turn formed macrophages and epithelioid cells. In the same year Yersin[6] inoculated rabbits with tubercle bacilli intravenously and studied tubercle formation in the liver and spleen from the 2nd through the 41st day. In his animals he described transformation of lymphocytic function as well as morphology as the lymphocytes accumulated in the infected area and became the cells known as epithelioid and giant cells.

Ruffer[27] (1890) described this process of lymphocyte change in the response of appendices and tonsils to normal bacterial invaders. His description of the

process needs no modern revision (his p 491) The (lymphocytes) nuclei are surrounded by a small amount of protoplasm of a pale rose color More careful examination of the lymphoid tissue reveals the presence of other mononucleated lymphocytes differing only slightly at first from the other cells surrounding them The nucleus of these cells is somewhat paler and its reticulum slightly more apparent though the protoplasm may not be at all increased in size as yet Other cells possess a nucleus which is again slightly larger paler and more vesicular than the latter as if the nuclear juice had increased at the expense of the solid part of the nucleus Lastly the nucleus and protoplasm become enormous and *the macrophage is formed* The macrophage is merely a lymphocyte which has assumed a new shape and a new function Excellent figures are appended to this paper

Metchnikoff's lectures[~] on the comparative pathology of inflammation delivered at the Pasteur Institute and published in 1892 demonstrated irrefutably the role of the lymphocyte as it hypertrophied to form first large mononuclears and then hematogenous macrophages

Arnold[~] (1893) introduced various foreign bodies into the mesentery and lymph sac of frogs and followed the gradual transitions of lymph cells to the large connective tissue elements in the ensuing aseptic inflammation Borrel[9] (1894) made similar observations in tubercles in the kidney of the rabbit Kanthack and Hardy[30] (1894-95) traced the origin of their hyaline cells (large mononuclear cells) to the lymphocytes of coelomic fluid and established them as morphological units distinct from the specifically granulated cells These investigators observed the same lymphocytic function in blister fluid from the skin of the arm of human volunteers into which *Bacillus ramosus* had been subsequently inoculated

Ranvier[31] (1900) studied the functions of clasmatocytes and leukocytes in aseptic inflammation of the peritoneum mesentery and appendix in the green frog salamander white rat guinea pig and rabbit He amply demonstrated that lymphocytes were a source of the defense elements

Maximow[32] (1902) in an extensive review of his experiments in aseptic inflammation described (1) the motility of lymphocytes (2) migration of lymphocytes from the smaller vessels into the field of inflammation and (3) their progressive hypertrophy into lymphocytogenous macrophages (his polyblasts) Maximow inserted a series of celloidin chambers into the connective tissues of rabbits dogs and pigeons More than 20 timed stages from 19 hours to 65 days of inflammation were so studied In 1903 the same investigator[33] extended his studies on new tissue growth in similar chambers from the fifth hour to the eight month in the process of scar formation He demonstrated that macrophages originated from newly emigrated lymphocytes and sessile clasmatocytes

In 1904 Maximow[34] while studying inflammatory connective tissue formation and changes in mast and fat cells of white rats found that in as short a period as four hours the hypertrophy of emigrated lymphocytes resulted in macrophage formation In the same year Ziegler[35] studied edema in the skin and subcutaneous tissues of 21 human subjects at autopsy He traced the gradual transformation of lymphocytes migrated from the blood vessels into

large phagocytic cells in areas of edema. Schwartz[36] (1904) introduced both glass and celloidin chambers into the connective tissue of rabbits and by the second hour, the small and larger lymphocytes were migrating from the vessels in appreciable numbers and between the 2nd and 10th hours, were transforming into large phagocytes.

Helly[37] (1905) injected a large variety of bacteria into the pleural cavities of more than 100 experimental animals and studied the resulting exudates at 24 and 48 hours. He observed a continuous series of transition forms between lymphocytes and macrophages. In the same year, Maximow[38] combined celloidin chambers inoculated with various bacteria with the study of aseptic chambers. The septic reactions differed only quantitatively, not qualitatively. He emphasized that the lymphocytogenous origin of the macrophages was found most convincingly in the early (fourth to eighth hour) stages of inflammation.

The thesis of the opposition, mostly Germanic at that time, to this concept of lymphocytic function was that the lymphocytes were incapable of locomotion and therefore could not migrate from the vessels into areas of inflammation and form macrophages. Lewis and Webster[39] however a decade or so later were to make painstaking demonstrations of lymphocytic locomotion.

Metchnikoff[23] (1905) in his second text clarified the nomenclature of such cells. He stated: The smaller white corpuscles found in fairly large numbers in the blood and lymph, commonly known as lymphocytes or small lymphocytes, are simply leucocytes with very little protoplasm, which in this state never fulfill phagocytic functions. It is only when it becomes older, when its nucleus, single and rich in chromatin, becomes surrounded by an ample layer of protoplasm, that the lymphocyte becomes capable of ingesting and resorbing foreign bodies. Several authors with Ehrlich at their head still assign to those larger cells the same name—lymphocytes. Others, however, give them the name of large mononuclear cells. Confusion is thus possible, especially as *Ehrlich includes under the same term the large mononucleated leucocytes* a very rare form in human blood which is distinguished by the greater staining capacity of its nucleus. To avoid this inconvenience I propose to designate the large lymphocytes by the name of blood macrophages and lymph macrophages (haemomacrophages, lymphomacrophages). . . . The mesoblastic phagocytes of the vertebrata are divided then into fixed phagocytes—the macrophages of the spleen, endothelia, connective tissue, neuroglia and muscle fibers—and free phagocytes. . . . The fixed macrophages and the free macrophages resemble one another so greatly that it is very often extremely difficult if not impossible to differentiate them. For this reason it is often very useful when the exact origin of a large phagocyte is not known simply to name it macrophage.

In the decade that followed there was abundant confirmation of an experimental nature of Metchnikoff's lucid statement. In 1906 Maximow[40] turned to the study of inflammation in cold blooded animals and chose the axolotl. His Taf XVII Fig 2 and Taf XVIII Fig 10 have been widely published in textbooks of pathology as classic portrayals of lymphocytic migration. In 1907 Zieler[41, 42] used a Finsen Reyn lamp's concentrated beam on the

inner surface of a rabbit's ear to study the resulting aseptic inflammation At 1 2 4, 15 and 24 hour stages he noted the migration of small lymphocytes into the areas of inflammation and their transformation through cytoplasmic changes into large lymphocytes or polyblasts In the same year Renaut[43] completed his extensive work on the potential phagocytes of the connective tissues which he called rhagiocrine cells He also worked on the source of the potential phagocytes in thoracic duct lymph He traced many of these phagocytic cells back to lymphocytes

The material of von Verebely[44] from the Surgical Clinic at Budapest consisted of granulating fatty tissue from patients with primary healed herniotomy incisions dead two to three weeks later of apoplexy pneumonia or pulmonary embolism as well as diverse patients with laparotomy incisions with primary healing or showing stitch abscesses and a long series of patients with excised tumors Von Verebely described the transformation of human lymphocytes into large mononuclear cells phagocytic for fatty debris in such areas Because of the surgical nature of his material quite frequently, he was able to date the exact onset of the initiating stimulus to the fatty tissues Stages studied were from 24 hours to 5 weeks in duration

In 1909 Fischer[45] introduced elder pith saturated with turpentine into the subcutaneous tissues of mice and rats and studied the sites of inflammation at 0 75 1 25 2 3 5 and 18 hour stages He too emphasized the necessity of early stage observation to catch the lymphocytes in process of migration from the smaller vessels

In the following year, Babkina[46] supplied a partial solution to the puzzling problem of the apparent sluggishness of lymphocytic acquisition of phagocytic powers in the lymphatic tissues themselves She imbedded celloidin tubules in the spleen lymph nodes and marrow of rabbits and observed that the local lymphocytes remained inert for a long time but that still later they hypertrophied and were transformed into phagocytic polyblasts (macrophages) She explained this sluggishness by pointing out that such tissues already contain large numbers of cells which were immediately phagocytic namely reticulum cells and that lymphocytic transformation into phagocytes in such areas is therefore seldom required

In 1911 Homen[47] studied tubercle formation within sciatic nerves of rabbits and guinea pigs He inoculated virulent human type tubercle bacilli into these structures and studied the reaction in serial stages from the sixth hour through the fifteenth month The origin of epithelioid cells from polyblasts and ultimately from lymphocytes as well as from fibroblasts and adventitial cells was apparent to him In the same year von Fieandt[48] injected virulent human tubercle bacilli into the internal carotid arteries of dogs and observed the transformation of lymphocytes into macrophages in the resulting tubercles in the meninges and cerebrum within 12 to 14 hours

Also in 1911 Wallgren * published careful descriptions of experimental hepatic tuberculosis in rabbits after injecting the organisms into cecal veins In two to four days the predominant lymphocytes showed numerous transitional stages to polyblasts In his Fig 1 Taf XIII eight stages in the development of the lymphocyte to epithelioid cell are depicted Here for the

first time, changes in the cell center, centrioles and attraction sphere were delineated, as progressive changes in lymphocytic function took place

In 1912 Downey and Weidenreich[50] studying the reaction of the lymphocytes of the appendix of the rabbit to the influx of intestinal bacterial flora, confirmed Ruffer's[7] findings with respect to the phagocytic transformation of the lymphocytes In 1913 Dubreuil[51] made an intensive study of lymphocytic functions He employed an unusual method in proving the derivation of the mononuclears from the lymphocytes in that he correlated concomitant granular mitochondrial changes as the lymphocytes changed function He also employed postvital staining with neutral red to show the same functional relationships of the lymphocyte

Aschoff[5] in his extensive contributions to our knowledge of a general defense system of cells excluded the lymphocytes from his scheme of a reticulo-endothelial system because, under the conditions in which his experiments were performed, he was uncertain as to whether lymphocytes were phagocytic for vital dyes Even while Aschoff was performing his work, Tschaschin[53, 54] was already demonstrating that small lymphocytes phagocytosed vital dyes until their cytoplasmic content was indistinguishable from ordinary macrophages Downey[5, 55] soon after demonstrated that the lymphocytes within the blood itself were capable of phagocytosing colloidal dyes if only the dyes were first made available to these cells by the simple expedient of double ligature of a vessel with study of the leukocytes in the interposed segment

Policard and Deplas[57] (1917) described the cellular constituents of normal and infected human granulation tissue These workers made counts of the cell types found at the surface at an intermediate level and at the depths of the granulations, at timed intervals from the 12th to the 25th day They described the manner in which lymphocytes migrated from the smaller vessels transformed into the macrophage and finally settled down as fibroblasts in the ultimate scar

The reports of Bergel[58] (1920) dealt with the lipolytic enzymes of the lymphocyte In one series of experiments he described the breakdown of the fatty constituents of the leprosy and tubercle bacilli within the cytoplasm of lymphocytes after the lymphocytes had ingested those organisms In another series in which various lipids were injected into the body cavities Bergel emphasized that the lymphocytes underwent structural changes as they assumed phagocytic and digestive functions toward lipids As this physiological function was exerted by the lymphocyte, its nucleus enlarged often becoming slightly indented a parallel change went on in the cytoplasm with gradual ingestion of one two or more fat droplets and the protoplasm of the cellular body also increased gradually As more and more droplets were ingested the cytoplasm became large the nucleus became plumper with a looser structure and the macrophage stage was attained

In the same year a comprehensive resume of Dominici's[4] experimental and observational works was published in three lengthy papers As a background for his views on the lymphocytic origin of the phagocytes he included a great deal of human material a scrotal fistula tertiary lesions of syphilis

mycotic infections and normal lymph nodes, as well as those with carcinomatous metastases

Latta[40] (1921) reported that *large lymphocytes in the lymphatic tissue of the intestine could take on abundant acidophilic cytoplasm and ingest lymphocytic remnants* In 1922 Danchakoff and Seidlin[41] injected a mass of the protein edestin into the mesenchymal tail plate of tadpoles In reference to lymphocytic functions the authors stated (p 105) As these cells advance toward the injected mass they exhibit a series of changes which made them rapidly acquire a digestive activity little expected from the small lymphocytes *A rapid transformation of a small lymphocyte into a histiotopic wandering cell* thereby takes place

In 1923 Maximow[42] added to the list of techniques by which the developmental potencies of lymphocytes had been studied Lymphocyte production of macrophages in tissue cultures of mammalian lymphoid tissue is well depicted (Taf XVIII 5 6 Taf XXI 13) In the same year Maximow[43] also investigated blood formation in the Selachii and found that histiocytic cells of the embryonic forms as well as of adults *arose from large lymphocytes which in turn arose from body mesenchyme* A year later this indefatigable investigator[44] had managed to produce tuberculosis in explants from mesenteric lymph nodes omentum and intermuscular connective tissue in tissue culture In the lymph nodes reticulum cells were of course the most active phagocytes However lymphocytes also played an active part hypertrophying and transforming into polyblasts and later sometimes acquiring an epithelioid character *joining the reticular cells in the process of tubercle and giant cell formation*

In the same year 1924 Alfejew[45] reported on the origin of the wandering cells of the connective tissues of the embryonic forms and newborn of many different species including man He concluded that the wandering cells arose from lymphocytes In 1925 Maximow[46] inoculated tissue cultures of buffy coats of the blood of rabbits and found that (p 429) the monocytes respond promptly to the stimulus and being larger and better prepared for the defense reaction sooner reach the fully developed epithelioid stage The lymphocytes are slower but nevertheless they follow in the same way and sooner or later join the monocytes in their transformation into epithelioid cells In the same year Timofejewsky and Benewolenskaja[47] reported similar findings In this year too Jordan[48] injected India ink into the dorsal lymph sac of frogs and concluded that the monocytes observed developed from lymphocytes

In 1926 Stillwell[49] injected India ink and fresh egg yolk into the tongues of adult live frogs and observed that the living mononuclear exudates were almost exclusively hematogenous in origin (p 90) They represent lymphocytes and monocytes of the circulating blood which have emigrated *out of the blood vessels and have undergone a rapid hypertrophy in the tissues*

In the same year Vierling[70] too studied living cells in Amphibian larvae He injected vital dyes into the heart or lymph vessels and then used the supravital dye neutral red to follow the changes in his preparations He saw that lymphocytes already tagged with the vital dyes which they had ingested within

the vessels migrated out of the blood and lymph vessels, and he concluded that these cells were capable of forming any of the elements of connective tissue.

In 1926 Lang[71] introduced bits of sponge containing lecithin and agar into subcutaneous tissues of animals into which he had already introduced India ink intravenously. In this way he demonstrated that although the endothelial cells at times contained intracellular carbon aggregates, they did not transform into phagocytic elements. Instead the macrophages arose from lymphocytes and monocytes. This work stressed that the hypertrophy of the emigrating lymphocytes and monocytes began while these cells were still *within* the capillary lumen. Transitional forms between lymphocytes and monocytes ordinarily missing or rare in the circulating blood seem to become common in the stagnant blood of the vessels (of the inflammatory site)."

In 1927, Timofejewsky and Benewolenskaja[7] repeated their previous work with tubercle bacilli, but at this time they used the cells of human blood. The lymphocytes and monocytes of man were likewise seen to transform into epithelioid cells.

In that same year, Michels and Globus[73] studied the lymphocyte reaction in the central nervous system of 12 human beings. Eight cases of polioencephalomyelitis and four cases of acute epidemic encephalitis were studied. The authors pointed out the favorable opportunity for determining the origin of perivascular infiltrations in inflammatory lesions in the central nervous system. There is a strong tendency for the inflammatory elements to be restricted *to the adventitial spaces for relatively long periods undergoing various* morphological changes.

Here in an inflammatory site almost devoid of mesenchymal elements the compound granular corpuscles (macrophages) were found to arise largely from lymphocytes and monocytes emigrated from the blood stream and to some extent from the mesenchymal glial elements as well. Because of the numerous lymphocyte migration pictures in some cases and their paucity in others one of their conclusions was that such cells must be extravasated in showers.

In 1928 Watson[74] studied phagocytosis in a case of histoplasmosis. Although reticulum cells were the chief source of phagocytes in the lymph nodes, spleen, liver, adrenals and lung, in the lymph nodes, liver and lung lymphocytes also became phagocytic for the yeastlike invaders.

In the decade 1915–1925 there was set forth the hypothesis by Mallory, McJunkin, Foot, Herzog and Marchand that simple endothelium lining the common *blood vessels gave rise to the monocytes of the peripheral blood and* to those cells they applied the name endothelial leukocytes. This group also believed that macrophages or histiocytes corresponded to these same endothelial leukocytes. As soon as the reticuloendothelial system had been delineated by Aschoff[63], Downey[60, 66, 71b] and Maximow[75] the hypothesis of endothelial leukocytes as a source of macrophages was abruptly abandoned for it became obvious that the littoral reticulum cells lining the blood or lymph spaces of the hematopoietic and sinusoidal organs were the only cells lining blood or lymph spaces which possessed true phagocytic and occasionally hematopoietic powers.

In the decade 1920–1930 there arose groups of investigators who empha

sized cell types other than lymphocytes and reticuloendothelial cells as the cellular origins of the macrophages One of these was von Mollendorf who claimed the fibroblast as the sole source of the phagocytes He denied the hematogenous origin of all exudative cells including neutrophilic leukocytes

Another group of investigators claimed the monocyte as the most important of the hematogenous macrophage sources The investigators who attached prior importance to the monocytes can be divided into two groups according to their interpretation of monocyte macrophage relationships Lewis[76] and Carrel and Ebeling[77] held that the monocyte could enhance its phagocytic powers and transform into the macrophage The other group has been called the supravitalists because its members have classified living blood cells according to the way they react to neutral red and Janus green Simpson[78] noted neutral red granule groups in the cytoplasm of the monocyte and believed they were characteristic of monocytes In 1925 Sabin Doan and Cunningham[79] distinguished two types of cells in the field of inflammation a clasmatocyte which they derived from the endothelium and a monocyte which developed in the spleen and in wide areas of the body from a primitive cell They explained that the monocyte of the tissues arises like a blood cell and has to undergo a maturation before it becomes a typical adult type They also stated of the monocyte that its remarkable power of cell division often makes it the predominating functional cell after it has time to mature

In the same year Cunningham Sabin Sugiyama and Kindwall[80] reported on the role of their monocyte in tuberculosis in rabbits They concluded that successive waves of nonphagocytic reticular cells and epithelioid cells appeared and reappeared in tuberculosis

In 1926 Murray Webb and Swann[81] reported on the appearance of a high monocytosis in the blood of many rabbits suffering from a disease from which they isolated the causative organism termed *Bacterium monocytogenes* (now *Listeria monocytogenes*) These workers performed six brief one stage experiments on the white cell response in pleural exudates They injected into the pleural cavities of successive normal rabbits peptone broth *B coli* and *L monocytogenes* At six hours they observed in all a predominantly polymorphonuclear leukocytic response Next they injected the same antigens into animals which were running high blood monocytoses due to systemic infection with *L monocytogenes* In these latter animals the response to peptone broth and *B coli* was as before but local injection of *L monocytogenes* elicited 30 4 per cent of large mononuclears at six hours Witts and Webb[82] (1927) utilized this organism in an attempt to establish the origin of monocytes They concluded that monocytes arose in the spleen and bone marrow

Bloom[83] (1928) made an extensive study of the origin of the monocyte in animals suffering with this disease using section supravital dry and wet smear techniques He concluded that the monocytes arose through transformation of lymphocytes occurring in areas of sluggish circulation such as the sinusoids of the liver and sinuses of the spleen and bone marrow

In the same year Bloom[84] studied focal necroses in rabbits which had been given large doses of a virulent strain of *L monocytogenes* intravenously These abscesses found in the liver spleen adrenals and lymph nodes consisted

largely of macrophages (his polyblasts) connected by a series of morphological transitions with blood lymphocytes and monocytes at the edges of the abscesses

Again, in 1928, Bloom[85] made supravital studies of the blood cells subcutaneous connective tissues and mesenteric lymph nodes of the white rat and found that lymphocytes monocytes and plasma cells all contained a segration apparatus of neutral red vacuoles and that such a configuration could not be used as a criterion in differentiating the agranular leukocytes By an ingenious method he stained the lymphocytes of the subcutaneous tissues intravascularly with neutral red, and these cells, while still within the vessel lumen, developed the segregation apparatus within their cytoplasm

In the same year, in tissue cultures of rabbit thoracic duct lymphocytes, Bloom[16] traced the lymphocytes through a 'monocyte stage to the macrophage and then to the fibroblast His supravital studies of such cells also revealed a neutral red rosette in the small early lymphocytogenous macrophages

Maximow[86] too utilized tissue cultures to study the leukocytes of the chick rabbit, guinea pig and *Macacus rhesus* He employed supravital stains, as well as customary histologic techniques To his tissue cultures he at times added inflammatory extracts Lithium carmine and neutral red Within four to eight hours the lymphocytes had eccentric nuclei increased cytoplasm, and increased pericentriolar neutral red vacuoles By 11 hours the limits between the lymphocytes and monocytes had been effaced and, just as in inflammation the lymphocytes had gradually hypertrophied to form elements which could not be distinguished from monocytes

Caffier[87] in the same year observed lymphocytic transformation into monocytes at 12 hours and later into histiocytes and epithelioid cells in his cultures of normal human blood cells Timofejewsky and Benewolenskaja[88] continued their work on lymphocytic formation of macrophages in 1928 reporting on the introduction of tubercle bacilli type BCG into their cultures They confirmed their earlier findings

Also in 1928 Lacassagne and Gricouroff[89] exposed *in vitro* cultures of rabbit blood leukocytes to the action of roentgen rays and radon These cells were observed at 2 4 5 5 7 12 24 29 and 53 hours on up to eight days Whereas beta rays rapidly destroyed the cells gamma rays in proper dosage permitted migration of lymphocytes and transformation of survivors into macrophages

Michels and Globus[90] (1929) in their second series of investigations described the transformation of lymphocytes and monocytes migrated from the blood stream in the encephalon of cases of paresis meningovascular syphilis and vascular syphilis Mitoses among the emigrating lymphocytes were frequently noted one of the few references found describing this process *in vivo* in inflammation

Stieve[91, 92] (1929) studied the uteri of more than 100 women in all stages of pregnancy during labor and the puerperium as well as nonpregnant human uteri He observed the lymphocyte as it progressively transformed into the histiocyte then the macrophage and ultimately the fibroblast or smooth

muscle cell both in the enlarging uterus of pregnancy with its physiological inflammatory process and in the early puerperium with its true local inflammation In the early stages of pregnancy, he observed the migration of the lymphocyte a readily available mobile source of the histiocyte, into the uterine tissue This process dwindled at the fourth month of pregnancy but was revived at the second day of the puerperium when again a mobile source of hematogenous macrophages was sorely needed to bolster the local defences of a uterine wall so recently subjected to the birth trauma

In 1929 Higgins and Palmer[93] injected autogenous blood into the subserous spaces of the stomach and cecum of rabbits They traced the development of blood monocytes and lymphocytes into histiocytic elements in the experimental hematomas so produced In the same year Kreyberg[94] cultured exudate cells from a case of tuberculous pleurisy Lymphocytes comprised 98 4 per cent of the agranular leukocytes, monocytes and macrophages the remainder The lymphocytes rapidly transformed into macrophages epithelioid cells Langhans giant cells and fibroblasts Supravital and vital staining techniques confirmed the observations made from stained smears In the same year Timofejewsky and Benewolenskaja[95] cultured mature and immature lymphocytes from five cases of lymphatic leukemia Both mature and immature lymphocytes were seen to develop into macrophages epithelioid cells and giant cells A year later Silberberg[96] confirmed the earlier work of Babkina [46]

In 1930 Sabin Doan and Forkner[97] studied the reaction of the connective tissues of the rabbit to chemical fractions derived from tubercle bacilli Their index of fractional activity was the stimulation of monocytes and their formation of epithelioid cells

In the same year Cappell[98] made an extensive study of the cytology of the inflammatory exudate of the peritoneal cavity He observed (p 433) By the end of six to nine hours the lymphocytic cells exhibit commencing neutral red storage coincidentally with an increase in the amount of cytoplasm After 12 hours while many cells of the lymphocyte type are present the majority of the mononuclears exhibit nuclear indentation a more abundant and basophilic cytoplasm and increased storage of neutral red the granules being in some aggregated in rosette form in others scattered throughout the cell No clear distinction can be made by supravital staining between lymphocytes monocytes and macrophages while typical examples of each can readily be identified the three types are connected by all intermediate stages and it is clear that a progressive development is going on in the smaller vessels by which they are rapidly acquiring the functional characters of young macrophages Cappell also obtained corresponding results after the use of vital stains

In 1931 Ekola[99] studied inflammation in the subcutaneous tissues of rabbits into which trypan blue sodium ricinoleate and diphtheria toxin had been previously introduced At timed intervals she was able to trace the origin of the macrophage from the blood lymphocyte the tissue clasmatocyte and the fibroblast

In 1932 Pierce[100] cultured human leukemic lymphocytes *in vitro* and observed that lymphoblasts produced monocytelike cells which in turn trans

formed into polyblasts (macrophages) In 1935, Seki[101] studied cells from the heart's blood of various reptiles and amphibia stained vitally with trypan blue and noted transitional forms between lymphocytes and monocytes

In 1936 Taliaferro and Cannon [102] in describing the cellular reaction in the spleen of Panamanian monkeys infected with malaria stated that (p 122) The increase in macrophages can be easily explained on the basis of their transformation from lymphocytes and our evidence is overwhelmingly in favor of such a conclusion ' As evidence they described an intense lymphocytopoiesis 'the cells in the activated follicles, particularly in the transitional zone look as if they were developing into macrophages After continued malarial stimulation the sinuses of the red pulp also show every gradation from lymphocyte to monocyte to monocyte with abnormal nuclear lobes to macrophages and somewhat the same picture is seen within the splenic cords In 1937 Taliaferro and Mulligan [103] studying malaria in Rhesus monkeys noted that the greatest number of new macrophages in spleen bone marrow, liver and adrenals arose from medium lymphocytes

In 1938 Hertzog[104] incubated blood of human patients suffering from acute and chronic lymphatic leukemia absolute and relative lymphocytoses and infectious mononucleosis with nonvirulent *Staphylococcus aureus* and *Streptococcus viridans* He demonstrated that lymphocytes even in their pre phagocytic stage showed some phagocytic activity and that phagocytosis was more marked in the leukocytoid lymphocytes of infectious mononucleosis with their larger cell bodies His photomicrographs of this process stand as objective evidence of this phase of lymphocytic function (his Figures 3 to 5)

In 1939 Kolouch[105] introduced a radically new technique for demonstrating the lymphocytic origin of macrophages After injection of egg white into the subcutaneous tissues of rabbits he made dry fixed spreads of the inflamed connective tissues at timed intervals and stained his preparations in the manner of blood smears This technique permitted accurate and detailed cytologic examination of the lymphocytes as they transformed into macrophages Kolouch pointed out that even with his improved technique inflammation must be studied within the first 14 hours or else the lymphocytic origin of the mononuclear cells is largely obscured

In 1940 Taliaferro and Kluver[106] made air-dried impressions of the liver spleen and bone marrow of Panamanian monkeys with malaria Intermediate stages between lymphocytes and monocytes and between monocytes and macrophages were increased In the same year Finlayson and Latta[107] studied the early reaction of the leptomeninges of the rabbit to injections of trypan blue at timed intervals They reported (p 286) it was found that within 30 minutes there occurred in the pia mater a perivascular infiltration of lymphocytes which gradually became transformed into monocytes and plasma cells Such cells in turn increased in size became more and more vacuolated and segregated increasing quantities of dye becoming typical macrophages

Also in the same year Plimpton[108] injected ventriculin subcutaneously into guinea pigs and rabbits Within three hours she observed lymphocytes hypertrophying into hematogenous macrophages (her polyblasts)

The works of Tompkins[109,112] concerning the role of large mononuclear leukocytes in inflammation will be considered as a separate topic in this monograph Her student Gray[113] in the same year (1940) followed the hematogenous origins of monocytes with differential supravital counts after injection of various synthetic triglycerides into the subcutaneous tissue of guinea pigs The large majority of these cells hypertrophied and became macrophages

In 1942 Berman[114] described a method for obtaining dry films from tissue cultures of rabbit lymph nodes He found transformation of lymphocytes to macrophages marked after 31 hours He stated Typical localized vacuolation seen in the lymphocytes of dry films is replaced by diffuse vacuolation at the time when both the directional polarity of the cell and the lymphocytic character of the nucleus are lost This accompanies a change in the type and location of pseudopodia and represents the point of transition from a lymphocyte to a polyblast of indifferent origin Berman was the first to emphasize that the lymphocyte as it transformed into the macrophage showed a progression of transitional modes of locomotion and pseudopodial formation

Clark Clark and Rex[115] and Ebert Sanders and Florey[116] had made use of windows or chambers in rabbit ears to observe leukocytic functions They were unable to observe the transformation of living lymphocytes into macrophages However in 1942 Harper[117] reported on fibrous wound healing as seen in transparent chambers inserted in the ears of rabbits He studied the process of wound healing from a period shortly after the production of the wound until the formation of the completed scar His pertinent conclusions were The formation of new cells and tissue back from the edges of the wounds by mitotic division and subsequent migration into the wound wa not observed to occur in these studies It was not possible to account for the fibroblasts or macrophages appearing in the healing area by mitotic division from pre-existing cells in the wound area A process of differentiation through which lymphocytes undergo progressive transformation into macrophages in the tissues at the wound edge was reported This process was observed to correspond closely to the differentiation of lymphocytes which is reported to occur in tissue culture

Frenchs[118] (1943) studied the influence of a monocytosis within the blood stream produced by *Listeria monocytogenes* upon the character of the cellular exudate He found a high percentage of polymorphonuclears at six hours in his controls but in his test animals the ratio of polymorphonuclears to monocytes was usually 1 to 1

In the same year Rey[119] in studying the reactions of the hamster associated with natural immunity to *Leishmania brasiliensis* noticed migration of lymphocytes and monocytes and their transformation to hematogenous macrophages in the third and fourth hours with peak activity in the 5th to 12th hours Destruction of the invading organisms by the hematogenous macrophages occurred between the 13th and 24th hours

In 1944 Dougherty[120] introduced the dry fixed impression technique to the study of inflammatory reactions of the brain and cerebrospinal fluid In addition he employed vital and supravital staining as well as ordinary section techniques He observed that (p 85) the first cells reacting to experi

mental brain wounds were lymphocytes which transformed to macrophages.'

In 1945 De Bruyn[121] studied the locomotion of lymphocytes cultured from the abdominal lymph nodes of rabbits and guinea pigs by means of motion pictures. The lymphocyte presented two phases: a locomotion phase in which the activity of the cells was polarized and a depolarized phase of activity. A gradual hypertrophy of the lymphocytes took place while concomitantly the depolarized phase of the lymphocytes changed progressively into the motion of macrophages which were continuously depolarized.

In 1949, Campbell and Good[122] studied the antigen antibody mechanisms in neurotropic virus diseases and observed lymphocytic transformation into intermediate polyblasts which, in turn, formed macrophages.

In the same year, Townsend and Campbell[123] utilized Kolouch's[105] technique in mice and rabbits to observe the effects of roentgen rays on the inflammatory cells of those animals. They reported (p. 1349): "Within two hours after the initial infiltration began, some lymphocytes were acquiring an increased nuclear and cytoplasmic volume which characterized early intermediate polyblast development. In the inflammatory site the evolution of the nuclei of the lymphocyte to that of the intermediate polyblast and finally to the macrophage could be followed in detail."

In 1950 Good[124] studied experimental allergic brain inflammation in rabbits, utilizing egg white as the antigen. In nonallergic inflammation in the brain he noted (p. 84): "Soon after their initial diapedesis the lymphocytes undergo changes in both their nuclear and cytoplasmic structure which eventually results in a transformation through the intermediate polyblast to large fat filled phagocytic macrophages of the inflammatory exudate." In the sensitized rabbits, there was some delay of the lymphocytic diapedesis but similar structural and functional changes eventuated.

In the same year Dougherty and Schneebeli[125] also making use of Kolouch's[105] technique, observed lymphocytic transformation into macrophages *in connective tissue spreads of the inflammatory tissue of the mouse.*

In summary, the transformation of monocytes and reticuloendothelial cells and their counterparts in the connective tissues appears to have been well established in the literature dealing with inflammation. The transformation of the lymphocyte to the macrophage has been reported to have been accomplished by the following morphologic and functional changes: increase in nuclear size, breaking up of coarse nuclear chromatin masses into fine angular pieces, assumption of a nuclear chromatin pattern with chromatin-parachromatin distinction, increase in parachromatin, increase in cytoplasm, increase in phagocytic ability for bacteria, cellular debris and vital dyes, increase in cytocentric size, increase in number of cytoplasmic neutral red vacuoles with aggregation into a rosettelike apparatus, and increasing evidences of depolarization locomotion.

It is the purpose of this investigation to report on a technical procedure which has permitted evaluation of the relative importance of the role played by lymphocytes and monocytes as macrophage producers in acute inflammation in man.

Materials and Methods

The site chosen for our tests in man is either the volar surface of the forearm or the anterior surface of the thigh although any other convenient region may be employed The skin is shaved and cleansed with alcohol Then by means of a sterile scalpel or razor blade the epithelium is scraped away from an area three or four millimeters in diameter When the papillary layer of the corium is reached fine bleeding points are in evidence If the lesion is further deepened bleeding increases and another site had rather be chosen A small amount of bleeding is to be desired as evidence that the corium has actually been reached

The time at which the epithelium is removed denotes the time at which the experimental inflammation begins because the trauma of the technique itself serves as an inflammatory stimulus A solution or suspension of the desired nonlethal inflammatory agent is next applied to the denuded corium with a platinum loop The lesion is then immediately covered with a sterile chemically clean cover slip which is surmounted in turn by a square of cardboard cut slightly larger than the cover slip The cardboard is covered by surgical adhesive tape measuring approximately two by four inches The tape is so applied that its center overlies the lesion If further tension is required leverage is obtained by placing a flat cork disc over the tape lying above the lesion and affixing the disc with a second and narrower piece of adhesive tape We have employed 15 mm square glass cover slips

For convenience in handling a hundred or so kits are prepared at a time each kit consisting of a cover slip and a corresponding cardboard square wrapped together in a paper wrapper These are sterilized in covered Petri dishes and can be removed individually as needed

The cells of the inflammatory exudate migrate to the undersurface of the cover slips flattening themselves out as they do so When this much has been accomplished (in 30 minutes to an hour depending on the nature of the inflammatory exudate) the cover slip is removed and rapidly air-dried At the same time another sterile cover slip is placed over the same lesion and the process is repeated as often as desired at timed intervals In this way a series of permanent fixed preparations of *in vivo* samplings of the cellular exudates of man have been obtained

The cover slips exhibit but a single layer of exudative cells on their under surfaces Having been rapidly air dried they are treated like a blood slide and stained with May Grunwald Giemsa or Wright Giemsa stain modified as follows about four to six drops of May Grunwald are put on the cover slip for one minute the stain is then diluted with the same number of drops of Haden s buffer solution and the mixture is allowed to remain for two minutes the mixture is then drained off and without washing six to eight drops of 1 5 strength Giemsa stain (1 5 drops Giemsa stain to each 1 0 cc of buffer) are added and allowed to remain six minutes The cover slip is then washed rapidly in distilled water blotted dry and mounted in Clarite on a slide

In staining the cover slips were either held in cover slip holders or mounted on an ordinary glass slide by means of a drop of rubber cement If the latter

method is employed the entire slide can be covered with the staining solutions and correspondingly larger amounts of the reagents are employed

Our photomicrographs (FIGURES 1 to 13) are demonstrations of cells which have migrated to the undersurfaces of such preparations Single lesions can thus be sampled for their cytologic content at short intervals throughout the first 48 hours of acute inflammation in man The preparations are permanent and their improved cytological detail permits careful comparison with the cells seen in ordinary dry smears of the blood or bone marrow aspirates

Forty two different lesions of this type were studied in healthy human volunteers and they composed over 250 separate cover slip preparations at 1 75 2 3 3 75 5 6 7, 9 10 12 14 15 16 16 5 18 20, 21, 22 23 24 28 31 40 and 47 5 hours of inflammation In addition to the trauma of the technique alone nonpyogenic antigens were employed (egg white diptheria toxoid triple typhoid vaccine old tuberculin and first strength PPD) to which the human subjects were not systemically immunized

That the participation of blood monocytes in local inflammation is in proportion to their numbers in the blood has been demonstrated previously in experimental animals by Murray Webb and Swann[41] and by Frenchs[118] Study of functional and structural changes in monocytes in acute inflammation in man was achieved by application of the technique described above to the study of a patient suffering from monocytic leukemia Naegeli type[1,5] a form of chronic granulocytic leukemia in which functionally maturated monocytes approximately 15 μ in diameter numbered more than 6 000 cells per cu mm (FIGURE 14) Prominent nuclear indentations tagged 85 per cent of these monocytes Our photomicrographs (FIGURES 15 to 18) depict cells which migrated to the undersurfaces of the preparations obtained at timed intervals from a single lesion inoculated with egg white

Concomitant high monocytoses in the peripheral blood of rabbits were produced by experimental *Listeria monocytogenes* infection Forty half grown male rabbits were used in this series Thirty five of these animals were used to establish the proper dosage and virulence of *L monocytogenes* required to obtain the monocytic conversion of the nongranular leukocytes of the blood Five animals were used for the actual experiments on local inflammation A strain of high virulence *L monocytogenes* American Type Culture was employed Its morphology was that of strongly Gram positive small straight or curved motile rods This strain produced leukopenia and death in rabbits within 15 hours after a single administration of 0 1 cc in 0 9 per cent sterile saline (No 1 nephelometer) from a 36 hour Difco-liver veal agar slant growth After four months passage on artificial media with monthly transplants on Difco-liver veal agar the virulence of this strain was reduced to levels at which 0 3 cc to 0 8 cc of our No 1 nephelometer reading diluted in sterile 0 9 per cent NaCl of a 36 hours growth on the same agar was then nonlethal when given intravenously At 60 to 100 hours a peak monocytosis was thus produced in the rabbit blood (FIGURE 19)

At the height of the above described monocytoses a local inflammation was produced on the rabbit s ear with appropriate modification of the technique described above for man as follows a tongue depressor was fitted as a splint

into the hollow of the inner side of the shaved ear and fixed in place by two strips of adhesive tape one encircling the ear at its base and the other two centimeters from its tip The outer ear of the rabbit then presented a smooth flat bare area whose vascular supply was in no way impaired The lesion was produced and studied in the center of this area in the manner described above for the volar surface of the forearm of man

Our photomicrographs (FIGURES 20 to 27) are demonstrations of cells which have migrated to the undersurfaces of such preparations Five rabbits with varying normal and converted blood pictures were tested as to their local inflammatory response to local *L monocytogenes* alone to the liver veal agar to avirulent *Staphylococcus albus* and to the virulent strain of *L monocytogenes*

Results

A Nonimmune acute inflammation in man This group consisted of 42 experimental lesions in which an antigen to which the human subjects were not systemically immunized was applied locally Experiments H 8 and H 11 are representative of this group and their protocols will be reported in detail

Experiment H-8 The lesion was prepared in the skin of the forearm of O E as described above and 0 05 cc of 1 1000 tuberculin (O T) and 0 05 cc of 1 per cent solution of trypan blue were applied to the lesion O E has had a consistently negative tuberculin test Blood samples were taken from O E before the start of the experiment and were within normal values In the next 24 hours seven consecutive cover slip preparations were obtained from this single lesion

At three hours neutrophilic leukocytes were present in great numbers FIGURE 1 is a photomicrograph of the 3 hour preparation from this lesion The neutrophils were larger than normal and appeared edematous Their cytoplasm was colorless and their specific granules were wide spaced A few intracytoplasmic vacuoles were present Moderate numbers of rounded up portions of neutrophilic leukocytic cytoplasm had broken away from the leukocytic cell body Such fragments were 6 to 8 μ in diameter Their specific granules were the same as those found within intact neutrophils A few eosinophils were present and a rare free portion of eosinophilic cytoplasm was noted

A few lymphocytes were likewise present at this stage Such a lymphocyte is depicted near the center of our FIGURE 1 This lymphocyte measures 11 $\times$ 12 μ and is swollen like the neutrophils although the latter in FIGURE 1 varied from 15 to as high as 20 μ in diameter The lymphocyte nucleus was composed of large dark chromatin masses poorly demarcated from scant colorless parachromatin The cytoplasm was sparse and consisted for the most part of basophilic spongioplasm Three or four azurophil granules were present in one area of the cytoplasm

A few macrophages with round oval or irregular nuclei were also present Their nuclei possessed a coarsely stippled or ringed chromatin pattern each chromatin piece being angular and irregular with frequent clumping of these pieces The colorless or light blue parachromatin was separate from the purple chromatin Nucleoli when seen were sparse irregular in outline and basophilic The cytoplasm was abundant with irregular outlines and con

sisted of a pale blue background containing mottled and vacuolated areas of almost colorless hyaloplasm Some of the vacuoles contained particles of ingested trypan blue These cells were the clasmatocytes or resting wandering cells of the connective tissue

At six hours neutrophilic leukocytes were still the most numerous of the cells They were smaller now measuring only 10 to 11 μ in their greater diameters The cytoplasm, in many areas peripheral to their nuclear lobes, was scant or missing Many more free cytoplasmic fragments were observed A few neutrophils possessed cytoplasmic vacuoles and some had ingested trypan blue

About a third of the exudative cells were lymphoid in character Many of these cells were small lymphocytes such as those shown in FIGURE 2 They measured from 7 to 11 μ in diameter These cells were frequently fixed in ameboid motion, and as a result the cellular outlines were irregular Intense activity was exhibited by the cytoplasmic nuclear interface of these small lymphocytes The lymphocytic nucleus which usually appears to be round in mears of peripheral blood possessed a more or less deeply grooved indentation near the cytocentrum of the cell body The nuclear membrane exhibited in addition irregularity of outline here and there along its entire extent The cytoplasm was deeply basophilic Some of the small lymphocytes contained ingested portions of the neutrophilic cytoplasm described above in their cell bodies The neutrophilic granules in such protoplasmic bits were acidophilic

Clasmatocytes too were greatly increased in number and frequently they exhibited kidney shaped or horseshoe shaped nuclear outlines bearing superficial resemblance to monocytes of the circulating blood of man although their cytoplasm contained no azurophilic dust There were occasional vacuoles and particles of ingested trypan blue but ingestion of bits of neutrophilic cytoplasm was infrequent in contrast to such activity of the small lymphocytes

At nine hours neutrophilic leukocytes made up about 50 per cent of the cells of the exudate The neutrophils showed profound changes Although a few were large and edematous the majority were shrunken Their cytoplasm was scanty and some were miniatures measuring only 7 μ in diameter Vacuolation of cytoplasm was sometimes noted The nuclear lobes had clumped Often the chromatin stained darker than normally and the chromatin masses showed evidence of clumping Free cytoplasmic portions of neutrophils were not much in evidence at this stage

The most striking change in the inflammatory picture at nine hours was an increase in the lymphocytes which could be found in this preparation FIGURE 3 depicts such a field Many of the lymphocytes in this field had been fixed in ameboid motion As a result they were wormlike in their elongated migratory pattern Although the more elongated forms rarely measured more than 15 μ in length they were correspondingly narrower in breadth (7 μ) An interesting additional feature of the nucleus other than its irregularity of outline was the breaking up of the coarse chromatin pieces ordinarily associated with the mature lymphocytic nucleus observed in the peripheral blood Although numerous coarse chromatin masses remained in the lymphocytic nuclei at this stage the parachromatin had increased in amount and was distinct in

some nuclear areas An examination of FIGURE 3 reveals that the cytoplasm is still scant, forming but a thin rim about the rounded or elongated nucleus In the actual specimen it was deeply basophilic flaky and occasionally vacuolated At times it contained ingested trypan blue particles as shown in one of the lymphocytes in FIGURE 3 Another one of the lymphocytes in FIGURE 3 presents a deep cleft in its nucleus The small size of this cell and its scant deeply basophilic cytoplasm characterize it as a lymphocyte

For ready comparison of such monocytoid lymphocytes with true monocytes of man as observed in his peripheral blood in the inset of our FIGURE 4 we have depicted a monocyte from the peripheral blood of O E from a smear taken at the beginning of this experiment The magnification is the same and the staining techniques were identical This monocyte was a large cell measuring 16 × 18 μ yet it was small among monocytes This larger size is an important distinguishing feature of the monocyte as opposed to the small phagocytic lymphocytes in these preparations The large cytoplasmic nuclear ratio of the blood monocyte is likewise apparent in the inset in FIGURE 4 The cytoplasm was a muddy blue gray and contained fine dustlike azurophil granules The nucleus showed a large nuclear bay The nuclear pattern was characterized by fairly coarse chromatin clumps and strands which nevertheless were well demarcated by distinct parachromatin Cells with all the characteristics of the cell shown in FIGURE 4 (inset) were rarely observed in our cover slip preparations taken from acute inflammatory lesions in subjects without increased monocytic populations in their peripheral bloods. FIGURE 5 (inset) depicts another monocyte from the blood of O E for further comparative study of these cell types

At 12 hours neutrophilic leukocytes comprised less than half the cells of the exudate As depicted in FIGURE 5 *the neutrophils are shrunken and their* nuclei were pyknotic and clumped and most of their cytoplasm peripheral to their nuclear lobes was missing They measured only 6 to 8 μ in diameter with a few of these cell types relatively more intact

The lymphocytes of the type depicted in FIGURE 4 which is a photomicrograph of this preparation when rounded up as they are in this figure measured only 10 to 12 μ in diameter Their small size and lymphocytic characteristics were much more apparent and resembled the well known characteristics of lymphocytes of the peripheral blood. They made up about 50 per cent of the cellular exudate at this stage The nucleus of these lymphocytes although not as yet enlarged showed increasing irregularity of outline and breaking up of denser chromatin masses into finer and finer distinct angular pieces This process more sharply delineated chinks of parachromatin (nuclear juice) which was apparently increased The cytoplasm in two or three of the more central lymphocytes in FIGURE 4 was slightly increased in amount This increase was probably more apparent than real for when viewed under the microscope this cytoplasm was bulging with ingested particles of trypan blue bits of neutrophilic cytoplasm and other types of cellular debris In other areas in this field lymphocytes with remnants of *neutrophilic nuclear lobes in their* cytoplasm were found occasionally

Clasmatocytes were as numerous as in the preceding stage but due to the

sisted of a pale blue background containing mottled and vacuolated areas of almost colorless hyaloplasm Some of the vacuoles contained particles of ingested trypan blue These cells were the clasmatocytes or resting wandering cells of the connective tissue

At six hours neutrophilic leukocytes were still the most numerous of the cells They were smaller, now measuring only 10 to 11 μ in their greater diameters The cytoplasm, in many areas peripheral to their nuclear lobes, was scant or missing Many more free cytoplasmic fragments were observed A few neutrophils possessed cytoplasmic vacuoles and some had ingested trypan blue

About a third of the exudative cells were lymphoid in character Many of these cells were small lymphocytes such as those shown in FIGURE 2 They measured from 7 to 11 μ in diameter These cells were frequently fixed in ameboid motion, and, as a result the cellular outlines were irregular Intense activity was exhibited by the cytoplasmic nuclear interface of these small lymphocytes The lymphocytic nucleus, which usually appears to be round in smears of peripheral blood, possessed a more or less deeply grooved indentation near the cytocentrum of the cell body The nuclear membrane exhibited in addition irregularity of outline here and there along its entire extent The cytoplasm was deeply basophilic Some of the small lymphocytes contained ingested portions of the neutrophilic cytoplasm described above in their cell bodies The neutrophilic granules in such protoplasmic bits were acidophilic

Clasmatocytes too were greatly increased in number, and frequently they exhibited kidney shaped or horseshoe shaped nuclear outlines bearing superficial resemblance to monocytes of the circulating blood of man although their cytoplasm contained no azurophilic dust There were occasional vacuoles and particles of ingested trypan blue but ingestion of bits of neutrophilic cytoplasm was infrequent in contrast to such activity of the small lymphocytes

At nine hours neutrophilic leukocytes made up about 50 per cent of the cells of the exudate The neutrophils showed profound changes Although a few were large and edematous the majority were shrunken Their cytoplasm was scanty and some were miniatures, measuring only 7 μ in diameter Vacuolation of cytoplasm was sometimes noted The nuclear lobes had clumped Often the chromatin stained darker than normally and the chromatin masses showed evidence of clumping Free cytoplasmic portions of neutrophils were not much in evidence at this stage

The most striking change in the inflammatory picture at nine hours was an increase in the lymphocytes which could be found in this preparation FIGURE 3 depicts such a field Many of the lymphocytes in this field had been fixed in ameboid motion As a result they were wormlike in their elongated migratory pattern Although the more elongated forms rarely measured more than 15 μ in length they were correspondingly narrower in breadth (7 μ) An interesting additional feature of the nucleus other than its irregularity of outline was the breaking up of the coarse chromatin pieces ordinarily associated with the mature lymphocytic nucleus observed in the peripheral blood Although numerous coarse chromatin masses remained in the lymphocytic nuclei at this stage the parachromatin had increased in amount and was distinct in

the structural and functional modification undergone by the lymphocytes in the earlier hours of the process (shown in FIGURES 1 to 5)

This experiment was concluded at the end of the first day At 24 hours the majority of the exudative cells were sessile macrophages with diameters up to 20 μ as depicted in FIGURE 7 One of the better preserved neutrophils mentioned in the preceding stage is also shown

Experiment H II The lesion was prepared in the skin of the anterior thigh of J R as described above and 0 05 cc of diphtheria toxoid was applied to the local lesion J R has had a consistently positive Schick test In the next 40 hours seven consecutive cover slip preparations were obtained from this single lesion Blood samples taken from J R revealed that he ran a monocytosis for short periods of from 9 to 10 per cent otherwise they were within normal values

At 2 hours a rim of red blood corpuscles at the lesion s edge was evidence of the previous trauma Neutrophilic leukocytes were present in great numbers They were edematous and measured up to 17 and 18 μ in diameter A few clasmatocytes eosinophils and lymphocytes were likewise observed The lymphocytic structure was identical with that of an ordinary medium sized lymphocyte as found in peripheral blood

At five hours a scattering of red corpuscles remained Neutrophils were the predominant cell type They had regained their normal size a few were phagocytic while others had early pyknotic changes in their nuclear lobes A few eosinophils were noted The clasmatocytes were increased in numbers In some areas such cells were found frequently with horseshoe shaped nuclei

The lymphocytes were also increased in numbers FIGURE 8 depicts two medium sized lymphocytes one of which was fixed while in motion amid a mass of neutrophils

At 12 hours neutrophils still comprised nearly half of the exudate cells Most of them were shrunken and had lost much of their cytoplasm peripheral to their nuclear lobes (FIGURE 9) The nuclear lobes themselves were frequently clumped and pyknotic Lymphocytes were likewise numerous and many of them were phagocytic FIGURE 9 depicts ten such lymphocytes demonstrating that even small lymphocytes measuring only 8 to 10 μ in diameter were capable of marked phagocytic activity One of the small lymphocytes in the center of the photomicrograph has ingested a portion of neutrophilic cytoplasm almost as large as its nucleus while another nearby has ingested a large portion of a pyknotic nuclear remnant

FIGURE 10 also taken from this same preparation illustrates the marked lymphocytic content of the cellular exudate at this stage Seven of these lymphocytes were quite small Four more showed slightly hypertrophied cytoplasm The nuclear membranes were usually irregular In many of the cells it can be seen that the coarser chromatin masses have been divided into finer angular chromatin pieces providing a distinct more prominent para chromatin Reference to the monocytes depicted at the same magnification in the insets to FIGURES 4 and 5 or actual measurement of the lymphocytes in our photomicrographs with a centimeter scale will assure our readers that the cells which we have designated as lymphocytes in these preparations were

great influx of lymphocytes evidenced at this period they were, numerically, relatively less important During the early stages of inflammation they were quite prominent and an important early source of macrophages but as the hematogenous defense elements continued to migrate into the area in increasingly large numbers the activation of most of the available clasmatocytes already having been accomplished, such clasmatocytic activity was gradually overshadowed by the hematogenous elements

At 14 hours pyknotic degenerated remnants of neutrophilic leukocytes comprised less than one fourth of the cells present One such remnant is depicted on the border of FIGURE 5 Although a noticeable number of clasmatocytes persisted the greatest number of cells present in this preparation were slightly hypertrophied lymphocytes like those depicted in FIGURE 5 Twelve lymphocytes are shown in this photomicrograph which like the remaining photomicrographs of this experiment was taken at the same magnification as the preceding figures Both nucleus and cytoplasm showed structural evidence of increasing activity Although four or five of the lymphocytes in this figure possessed typical lymphocytic nuclei with large coarse chromatin massed, the *remainder presented irregularity of nuclear outline and smaller broken up* chromatin particles Cell diameters now ranged from 11 to as high as 13 μ The cytoplasm was more abundant than in the preceding stage Occasional large clear intracytoplasmic vacuoles made their appearance The cytoplasmic increase was due to parallel increases in both basophilic spongioplasm and colorless hyaloplasm The basophilic material seemed to be arranged as delicate network against a colorless background The cytoplasmic outline was indefinite, and numerous thin delicate processes protruded from its periphery

At 21 hours there was evidence of a second migration of structurally intact neutrophilic leukocytes in moderate numbers Although a number of the macrophages or histiocytes in this preparation still presented structural evidence of their lymphocytic origin as described above for the 14-hour stage the majority of these cells now resembled those of our FIGURE 6 a photomicrograph of eight such macrophages from this preparation These cells possessed diameters of from 14 to 17 μ There had been an increase in the size of both nucleus and cytoplasm The nucleus was round oval or slightly indented It was larger than in the preceding stage The chromatin pieces were small angular, and irregularly distributed They left distinct irregular parachromatin spaces increased in extent as though the nuclear juice increased in quantity, had widened the chromatin interstices The cytoplasm too was much more abundant than in the preceding stage and this latest increase was due largely to a rather diffuse increase in the colorless portion of the cell body One of the cells in FIGURE 6 presented a nucleus with a deeper indentation than the others and thus superficially resembled the monocyte in FIGURE 4 (inset)

Observation of this preparation at the 21 hour stage emphasized the need for careful evaluation of the structural features of the mononuclear exudative cells, in general during the first 12 to 14 hours of acute inflammation If the inflammatory process had been observed for the first time beginning with the 21 hour stage represented by FIGURE 6 there would have been little to indicate

the structural and functional modification undergone by the lymphocytes in the earlier hours of the process (shown in FIGURES 1 to 5)

This experiment was concluded at the end of the first day At 24 hours the majority of the exudative cells were sessile macrophages with diameters up to 20 μ as depicted in FIGURE 7 One of the better preserved neutrophils mentioned in the preceding stage is also shown

Experiment H 11 The lesion was prepared in the skin of the anterior thigh of J R as described above and 0 05 cc of diphtheria toxoid was applied to the local lesion J R has had a consistently positive Schick test In the next 40 hours seven consecutive cover slip preparations were obtained from this single lesion Blood samples taken from J R revealed that he ran a monocytosis for short periods of from 9 to 10 per cent otherwise they were within normal values

At 2 hours a rim of red blood corpuscles at the lesion s edge was evidence of the previous trauma Neutrophilic leukocytes were present in great numbers They were edematous and measured up to 17 and 18 μ in diameter A few clasmatocytes eosinophils and lymphocytes were likewise observed The lymphocytic structure was identical with that of an ordinary medium sized lymphocyte as found in peripheral blood

At five hours a scattering of red corpuscles remained Neutrophils were the predominant cell type They had regained their normal size a few were phagocytic while others had early pyknotic changes in their nuclear lobes A few eosinophils were noted The clasmatocytes were increased in numbers In some areas such cells were found frequently with horseshoe shaped nuclei

The lymphocytes were also increased in numbers FIGURE 8 depicts two medium sized lymphocytes one of which was fixed while in motion amid a mass of neutrophils

At 12 hours neutrophils still comprised nearly half of the exudate cells Most of them were shrunken and had lost much of their cytoplasm peripheral to their nuclear lobes (FIGURE 9) The nuclear lobes themselves were frequently clumped and pyknotic Lymphocytes were likewise numerous and many of them were phagocytic FIGURE 9 depicts ten such lymphocytes demonstrating that even small lymphocytes measuring only 8 to 10 μ in diameter were capable of marked phagocytic activity One of the small lymphocytes in the center of the photomicrograph has ingested a portion of neutrophilic cytoplasm almost as large as its nucleus while another nearby has ingested a large portion of a pyknotic nuclear remnant

FIGURE 10 also taken from this same preparation illustrates the marked lymphocytic content of the cellular exudate at this stage Seven of these lymphocytes were quite small Four more showed slightly hypertrophied cytoplasm The nuclear membranes were usually irregular In many of the cells it can be seen that the coarser chromatin masses have been divided into finer angular chromatin pieces providing a distinct more prominent parachromatin Reference to the monocytes depicted at the same magnification in the insets to FIGURES 4 and 5 or actual measurement of the lymphocytes in our photomicrographs with a centimeter scale will assure our readers that the cells which we have designated as lymphocytes in these preparations were

well under 14 μ in diameter Neutrophilic leukocytes however, cannot be used for size orientation in such preparations at any time Early in acute inflammation in man they appeared to be edematous and enlarged Soon afterwards, they diminished in size, gave up portions of their cytoplasm as broken off cytoplasmic fragments and ultimately degenerated In many of these preparations, some lymphocytes have become shrunken were almost devoid of cytoplasm and, at times even possessed pyknotic nuclei An occasional lymphocyte was thus unable to survive in the field of inflammation even when the irritant was a relatively mild one such as we have employed

At 14 hours the neutrophils presented the same features as described in the preceding stage Several of them are depicted in FIGURE 11 Lymphocytes now comprised almost half of the exudate cells FIGURE 11 depicts 12 lymphocytes, many of them slightly hypertrophied There has been a slight increase in the parachromatin of the nucleus distinctly separating the angular finer chromatin pieces In addition the cytoplasm was more abundant due to a diffuse increase in colorless hyaloplasm vacuoles and ingested material One of the smallest lymphocytes shown in FIGURE 11 which still possessed the typical pachychromatic nuclear pattern has ingested a red corpuscle The cytoplasmic outline of several of the larger lymphocytes in FIGURE 11 presented numerous blunted pseudopodia An occasional lymphocyte (not depicted) has ingested a large portion of neutrophilic cytoplasm Clasmatocytes were numerous but there had been but little addition to their numbers since the early stages None of the cells of the inflammatory exudate observed in any of our preparations exhibited any evidence of mitotic activity or cellular division

At 16.5 hours the lymphocytes were so hypertrophied that they were no longer clearly distinguishable from histogenous macrophages which were also present Degenerating neutrophils comprised about a third of the cells at this stage FIGURE 12 depicts a representative field from this preparation Two of the exudative mononuclears were still obviously lymphocytes The remainder had increased amounts of cytoplasm and increased amounts of nuclear parachromatin so that they were designated as lymphocytogenous macrophages or simply macrophages The chromatin pieces were fine and angular and irregularly distributed and there was chromatin parachromatin distinction One of these macrophages had ingested a large nuclear remnant These larger cells had long diameters of from 15 to as high as 22 μ or more One of these cells on the border of the field has ingested a portion of neutrophilic cytoplasm

At 28 hours there was evidence that a second wave of neutrophils had appeared in the field at some time after the preceding stage Almost all the mononuclear cells were macrophages A group of these is depicted in FIGURE 13 They are particularly phagocytic for bits of neutrophilic cytoplasm Their structure at this time afforded no clue to their lymphocytic origin as depicted in FIGURES 8 to 12 Basophilic leukocytes were occasionally observed with fairly intact structure at this time

At 40 hours macrophages predominated The remaining degenerated neutrophils were being phagocytosed *in toto* A single binucleate giant cell was found in this preparation

The succeeding experiments of this group presented similar findings except for the cellular responses to the trauma resulting from the technique alone In this latter group, the leukocytic cycles were of less intensity and more transient than when an antigenic stimulus was applied Although there was an abundance of neutrophils there was a less marked response of histogenous macrophages and a minimal response of lymphocytogenous macrophages Apparently, in the absence of antigenic stimulation a mobile source of macrophages was not needed to accomplish the simple removal and reparative functions required for the healing of the experimental lesion itself

B *Acute inflammation in man with concomitant monocytosis* The patient studied suffered from a form of chronic granulocytic leukemia (monocytic leukemia Naegeli type)[1,6] in which in addition to developing granulocytes, functionally maturated monocytes numbered more than 6 000 cells per cubic mm (FIGURE 14) These monocytes were approximately 15 μ in diameter and 85 per cent of them possessed prominent nuclear indentations which served to 'tag' them in the field of inflammation Serial preparations stained like blood smears were obtained at timed intervals, from a single lesion inoculated with egg white

At four hours monocytes were present in small numbers in the field of inflammation and were phagocytic and slightly hypertrophied (FIGURE 15) At seven hours their numbers were increased (FIGURE 16) At times their nuclei showed polymorphous changes with only thin strands connecting individual lobes but amitotic division was not in evidence At 12 hours the monocytes were the predominant mononuclear cell They were further hypertrophied (FIGURE 17) and had ingested neutrophilic cytoplasmic fragments At 12 75 hours the numerous monocytes approximately 18 μ in diameter were identifiable as monocytogenous macrophages (FIGURE 18)

Structural modifications accompanying transformation of blood monocytes (FIGURE 14) into monocytogenous macrophages (FIGURES 15 to 18) were increase in cytoplasm particularly hyaloplasm gradual diminution of the azurophilic granulation characteristic of the monocyte in man and increase in nuclear size in parachromatin and in distinctness of chromatin network with *pronounced accentuation of the nuclear indentations* Hypertrophying monocytes an important mobile source of macrophages in this patient with a marked monocytosis in his peripheral blood at key stages of transformation were hour for hour 6 μ larger than comparable lymphocytes (cf FIGURES 1 to 5 and 8 to 11)

C *Acute inflammation in rabbits with concomitant monocytoses* Experiment L-40 is representative of this group and its protocol will be presented in detail Before experimentation the blood of this half grown male rabbit was shown to be normal Next 0 8 cc of a 5 cc suspension of the growth of one plain agar and one liver agar slant (24 hour) of a virulent strain of *L monocytogenes* was injected intravenously into this animal The blood was checked 44 hours later and a sharply rising monocytosis was noted At this time the monocytes comprised 21 per cent of all the white blood cells and numbered 2 600 per cu mm

A denuded area of the ear was prepared as described above and was swabbed

with a 70-hour culture of the same virulent *Listeria* strain suspended in sterile physiological saline A successful attempt was made to obtain the peak blood monocytosis during the time of study of the local inflammation

At four hours, red cells were seen indicative of minute traumatic hemorrhage in the field Many polymorphonuclears (rabbit heterophils) had already started their migration At seven hours the inflammatory site showed large numbers of intact and ameboid heterophils Some were vacuolated others were losing their granules and the cytoplasm of some was swollen Lymphocytes were not infrequent They had accumulated more hyaloplasm and in some cases, their nucleus was larger, while their chromatin pattern became a little more distinct A few lymphocytes were in process of hypertrophy but the majority still had the appearance of blood lymphocytes A few macrophages were also observed

At eight hours, the blood was re examined and the monocytes had now increased to 5,250 per cu mm An extremely low lymphocyte percentage (7 per cent) should also be noted At 9 hours, the heterophils of the exudate were swollen with cytoplasmic and nuclear vacuoles The cells themselves were clumped and disintegrating Some of their nuclei had lost their staining affinity The lymphocytes also present, showed an increase in hyaloplastic areas The nucleus showed separation of chromatin from increased parachromatin and the nuclear membrane appeared to be thinner 'Monocytoid cells and monocytes or their derivatives were lacking

At 12 hours, many of the numerous heterophils present were still intact, but others were clumped and in various stages of disintegration Small lymphocytes were present but they were hypertrophying A few macrophages had already developed

At 16 hours, the blood showed a peak monocytosis for this animal of 7 300 monocytes per cu mm (FIGURE 19) In the lesion itself ordinary small lymphocytes had hypertrophied still further Small early macrophages and larger macrophages were showing signs of phagocytic activity Very few cells with slightly indented nuclei (monocytoid cells) were seen

At 22 hours the heterophils were in clumps some had disintegrated while groups of others had been invaded by the large lymphocytogenous macrophages with their customary round oval or slightly irregularly shaped nuclei A small number of the lymphocytogenous macrophages were monocytoid that is, they had slightly indented nuclei At this time the first few monocytes (FIGURE 20) made their appearance at the site of inflammation Note that the chromatin masses already showed a slight tendency to splitting and that vacuoles were beginning to form in the cytoplasm

At 26 hours more monocytes had taken their place alongside the lymphocytogenous cells The heterophils had all but disintegrated Great numbers were amorphous poorly stained clumps Many had been ingested by the numerous lymphocytogenous macrophages Lymphocytes were still found in small numbers By the time the monocytes had reached the under surface of the cover slip their nuclear pattern had undergone a slight breaking up of its clumps and strings of chromatin into small irregular angular pieces A concomitant increase of parachromatin in the monocytes tended to distend the

nucleus so that eventually a fine nuclear pattern with sharp chromatin parachromatin distinction was reached which resembled the pattern of the lymphocytogenous macrophage or reticuloendothelial cells nucleus The monocytic nucleus retained its characteristic horseshoe shape and as the parachromatin of the nucleus continued to increase the large nuclear bay was even accentuated This facilitated the identification of these cells

At 31 5 hours the monocytes had increased in number They along with the lymphocytogenous macrophages were beginning to take on the characteristics of large macrophages The chromatin of the monocytes was splitting up from the coarser arrangement seen in the blood the parachromatin was distinct and on the increase and the amount of cytoplasm had increased, especially the hyaloplastic and vacuolar portions FIGURE 21 shows three such monocytes whose nuclei are still greatly indented Two macrophages of either lymphocytogenous or histiocytic origin are also shown in FIGURE 21 to aid in the comparison of the two-cell types

At 41 hours the heterophils were of interest only in showing further disintegration disappearance or phagocytosis by the macrophages Lymphocytogenous and histogenous macrophages were present and were large and active Often they had banded together to form giant cells in which the nuclei were all oval round or slightly irregular in shape just as Maximow[37] had described them in 1902 In addition there were many monocytes present hypertrophying into monocytogenous macrophages and this hypertrophy tended to dominate the picture FIGURE 22 shows a monocyte from the lesion at this time and two monocytogenous macrophages Their nuclei are characteristically deeply indented The small cells in the figure were disintegrating heterophils These monocytogenous macrophages comprised an important portion of the macrophage cell group Blood studies taken at these later stages showed a continual fall in both absolute and relative monocyte counts

At the same time another phase had begun in which the large histogenous and lymphocytogenous macrophages arranged in groups had settled down after digesting their phagocytosed particles Long processes extended from their cytoplasm and their cell outlines had a stellate appearance Their vacuoles were small sharply outlined and regular in size and distribution The remainder of their cytoplasm was more basophilic and homogeneous At the same time the chromatin pattern of their nucleus had become more finely reticulated In 1902 Maximow[37] had described such a phenomenon in the process of scar tissue formation *in vivo* and later he made a similar observation in his tissue culture experiments (1928)[38a]

At 56 hours entire groups of monocytogenous macrophages were assembled with the nonmonocytic forms FIGURE 23 shows four such monocytogenous macrophages With their increased phagocytic activity they had attained huge proportions Their finely stippled chromatin pattern was similar to that of ordinary macrophages but in each case the horseshoe shaped indentation of the nucleus has remained a constant and even accentuated feature A nonmonocytic macrophage is shown for comparison in FIGURE 23

At this stage the monocytogenous macrophages exhibited two further potentialities they banded together with similar cells to form purely mono-

cytogenous giant cells and with nonmonocytic macrophages to form hybrid giant cells

At 57 hours the phagocytic giant cells were present in three forms among the numerous monocytogenous macrophages They were of the ordinary lymphocytogenous or histogenous type, all of the nuclei being round or oval, or they were made up of purely monocytogenous macrophages with all their nuclei deeply indented or they were hybrid and represented the fusion of histogenous or lymphocytogenous components with monocytogenous components FIGURE 24 represents such a hybrid giant cell found at this time The one monocytogenous component, with its indented nucleus, stands out from the three nonmonocytic components also making up the cell

At 70 hours, the macrophages and their derivatives dominated the picture As a large portion of these cells were monocytic in origin, our remaining descriptions will be confined to the part they played In addition to many purely monocytogenous macrophages which were present in large clumps purely monoctyogenous giant cells or hybrid giant cells (FIGURE 25) were seen From this field, the figure portrays a typical phagocytic hybrid giant cell with one monocytogenous and one nonmonocytic component Such cells add by the distinction of their nuclei to the concept that fusion of macrophages is at least one important method of giant cell formation

The monocytogenous macrophages exhibited still further potentialities through formation of monocytogenous clasmatocytes (FIGURE 26) with elongated *sessile processes and very fine chromatin nuclear pattern, the nucleus* still retaining its large indentation They even approached the fibroblast stage depicted in FIGURE 27 with its outstretched stellate processes fine and regular vacuolization and characteristic nuclear indentation This cell represents a transitional stage between a monocytogenous clasmatocyte and a fibroblast

At 98 hours the cytology was such that both lymphocytogenous and histogenous macrophages could still be distinguished from the monocytogenous forms The giant cells were present in the three forms found in this lesion

Control lesions testing the inflammatory response to the liver veal agar avirulent *Staphylococcus albus* and to local but not systemic administration of *L monocytogenes* revealed lymphocytogenous and histogenous formation of macrophages as described by Kolouch[105] but a relatively sparse monocytic origin In this respect our FIGURES 20 to 27 should be compared with Kolouch s *figures 3 to 6 depicting comparable control lymphocytogenous formation* of macrophages in acute inflammation in the subcutaneous connective tissues of the rabbit

Discussion

The transformation of the lymphocytes of man into macrophages proceeds in an orderly fashion in the simple cycle of acute inflammation When the exciting antigen is one to which the human subject is not systemically immunized migration of the lymphocytes and early transformational changes reach their peak between 12 and 14 hours At this same stage the lymphocytes and their hypertrophied forms are usually numerically in excess of

the exudative neutrophils for the first time in the cycle This transformation of lymphocytes into macrophages involves modifications of a functional and structural nature in both lymphocyte nucleus and cytoplasm These modifications are usually so advanced by the 14th to 16th hour of inflammation that the lymphocytic origin of the macrophages is largely obscured at any later stage in the dynamic process

In our present report, by means of an original technical procedure the cellular exudate of single lesions in man has been sampled hour by hour In a large series of experiments cover slip preparations dried and stained like blood smears were obtained which allowed detailed comparison of the cells obtained in acutely inflamed tissues of man with the cells of blood smears In this same series we have noted mean structure and function of large numbers of lymphocytes of single lesions concurrent with the chronological progression of inflammation In a simple nonimmune cycle in from two to nine hours the lymphocytes were present as lymphocytes but in ever increasing numbers In from 9 to 14 hours the great mass of lymphocytes presented cytoplasmic and nuclear hypertrophy In from 14 to 18 hours lymphocytogenous macrophages dominated the field There was no need to resort to the study of intermediate forms or transitional stages within the cells of a single sampling It was the mass of lymphocytes that changed progressively hour by hour It was the structural and functional condition of the majority of the lymphocytes in each preparation during each stage of inflammation that most concerned us This description can be substantiated by a review of FIGURES 1 to 13 which present liberal portions of the cellular exudates of single lesions in consecutive stages

The majority of the lymphocytes studied in each stage progressively changed in structure and function until a stage was reached at which the majority of the mononuclear cells were no longer lymphocytes as such but were larger phagocytic forms It is also true that in most preparations there were some lymphocytes which lagged behind the majority and remained unaltered The contrasting possibility cannot be excluded that some lymphocytes may be capable of precocious transformation and of anticipating their more slowly changing but preponderant fellow lymphocytes To demonstrate the lymphocytic origin of the macrophages *in vivo* then attention has been directed to gradual alterations in the majority of the lymphocytes in each lesion Little mention has been made therefore of the occasional lymphocytes which were out of phase with the functional conditions of the greater number of their fellow exudative cells

Of fundamental importance is the problem of relating physical and chemical alterations in the lymphocyte nucleus and cytoplasm to their structural and functional modifications as the transformation into the macrophage proceeds Furthermore any understanding of alterations within the cell presupposes a clear knowledge of the functional relationship of the lymphocyte to the changing conditions of its environment

One of the first structural modifications which the lymphocytic nucleus undergoes as it transforms into a macrophage nucleus is a change in the size and distribution of its chromatin The lymphocyte as it reaches the field of inflammation usually possesses a nucleus which is pachychromatic the chro

matin pieces are coarse and large, the parachromatin or nuclear juice content of the nucleus is scanty Furthermore, the parachromatin, which is colorless in Romanowsky stains, is poorly delimited from the chromatin masses whose border blend imperceptibly with the parachromatin Within 9 to 12 hours, and often before this period the large chromatin pieces which stain deeply basophilic are observed to be divided into smaller and smaller irregular, angular pieces At first, this process is accomplished with only a relative increase in parachromatin but, as the process of chromatin subdivision proceed there is an actual increase in the colorless parachromatin At the same time the smaller, angular chromatin pieces begin to form an irregular but definite chromatin network, and the parachromatin becomes more and more distinct from this chromatin framework This nuclear change may precede or accompany cytoplasmic modifications

The next nuclear modification which may be noted is increasing irregularity of the shape or outline of the nucleus Even the lymphocytes of the blood when observed in wet smears possess a nuclear indentation[127] In the field of inflammation, this lymphocytic indentation is usually noticeable as the cleft becomes deeper in the outstretched cell Changes in the nuclear membrane are not confined to the region of the widening indentation Other areas of nuclear membrane at some distance from the indentation, show definite undulation suggestive of increased activity at the nuclear cytoplasmic interface If vacuoles or ingested substances are in contact with the nuclear membrane they are able to indent the nucleus at the point of contact suggesting again an increased fluidity of the nucleus By the 14th hour the nucleus of the lymphocyte has increased in size This increase is apparently due to the increased amounts of nuclear juice or parachromatin which latter becomes increasingly distinct (FIGURE 11)

Little is known concerning the functional changes in the lymphocytic nucleus A beginning in this direction has been made by Thorell[128] who in studying metabolism in the lymphocytes found that the intracellular nucleic acid metabolism of large lymphocytes indicated a high intensity of growth

Lymphocytic cytoplasm undergoes more apparent structural and functional changes The most remarkable and earliest of these changes in lymphocytic cytoplasm is the assumption of the phagocytic function (*cf* FIGURES 4 and 9) This ability is possessed by lymphocytes within the blood stream but is usually a latent one Downey[55, 56] demonstrated that lymphocytes within the blood itself possessed the ability of phagocytosing vital dyes if the dyes were made available to these cells by the simple expedient of double ligature of a vessel followed by study of the lymphocytes in the interposed segment Included in these phagocytic powers then is the ability to ingest vital dyes (*cf* experiment H 6)

Equally rapid in its assumption by the lymphocyte in the area of inflammation, is the ability to form a rosette of numerous neutral red vacuoles in the cytoplasm of the [illegible]lear bay This property of the lymphocytes has been demonstrated b[illegible][86a] Bloom,[86] Hall[1 9 130] and Cappell[98] Interestingly enough [illegible]ytes also become peroxidase positive[131] and Nadi positive[132] [illegible]m in the field of inflammation

The cytoplasm gradually increases in size until the large cell body size of the macrophage is attained In this connection it is pertinent to recall Lang s studies of aseptic inflammation in the subcutaneous tissue of rabbits Lang[71] observed that this hypertrophy of the emigrating lymphocytes began while they were still within the capillary lumen He had previously injected India ink intravenously into his animals He observed that the hypertrophied cytoplasm of lymphocytes in the stagnant blood of the vessels of the inflam matory site began to ingest small particles of India ink Probably, many of the early functional and structural modifications of the lymphocyte as it grows into a macrophage, have already begun or are capable of beginning within the capillary lumen at the inflammatory site The increase in lymphocytic cytoplasm is probably due fundamentally to a gradual increase in hyaloplasm as described by Kolouch[103] although occasionally an equal increase in basophilic spongioplasm is also noted Jones[133] has furnished evidence that the hyaloplastic portion of blood cell cytoplasm represents mitochondria The cytoplasm of the lymphocytes undergoes further enlargement of course through phagocytosis of protein antigens red blood corpuscles and fragments of free neutrophilic cytoplasm

The observation of ingestion of cytoplasmic fragments of neutrophilic leukocytes was made possible by the clarity of the cellular details reached by a technique which permitted study of the tissue exudate dried and stained like a blood smear This relationship between hypertrophying lymphocyte and shedding neutrophilic cytoplasm may afford amino acid residues lipids carbohydrates and other building stones needed by the lymphocytic cell body in its rapid and relatively great cytoplasmic structural reorientation and growth preparatory to the assumption of its macrophage function The changes in the submicroscopic particulates mitochondria micellae and Golgi net as the lymphocyte tran forms into the macrophage await further studies with the phase and electron microscope Changes in lipids and carbohydrate content await the application of methods as utilized by Gomori[134] and Wislocki and Dempsey[135]

With reference to cytoplasmic organelles it is interesting to recall Wallgren s[49] observations on the growth of the cytocentrum as the lymphocytes transformed into macrophages His Fig 1 b d g h Taf XIII depict the customary two centrioles in the cytoplasmic bay of the lymphocyte As the lymphocyte hypertrophied at first three centrioles later six or seven were present in the same area finally as the macrophage stage was attained 12 or so centriolar bodies were present Concurrently astral rays were elaborated and ran centrifugally from the aggregated centrioles

Early opposition to Metchnikoff s concept of the lymphocytic origin of the macrophage was based on the mistaken view that lymphocytes were not motile and therefore were incapable of migration Later differences in modes of locomotion were seized upon to differentiate lymphocytes from other cellular sources of macrophages However Berman[114] had observed a gradual transition from lymphocytic to macrophage pseudopodial activity but in fixed preparations De Bruyn[1 1] made motion picture studies of lymphocytes as they hypertrophied toward the macrophage stage Their mode of locomotion

matin pieces are coarse and large, the parachromatin or nuclear juice content of the nucleus is scanty. Furthermore, the parachromatin, which is colorless in Romanowsky stains, is poorly delimited from the chromatin masses whose borders blend imperceptibly with the parachromatin. Within 9 to 12 hours and often before this period the large chromatin pieces which stain deeply basophilic are observed to be divided into smaller and smaller irregular, angular pieces. At first, this process is accomplished with only a relative increase in parachromatin but, as the process of chromatin subdivision proceeds there is an actual increase in the colorless parachromatin. At the same time, the smaller, angular chromatin pieces begin to form an irregular but definite chromatin network, and the parachromatin becomes more and more distinct from this chromatin framework. This nuclear change may precede or accompany cytoplasmic modifications.

The next nuclear modification which may be noted is increasing irregularity of the shape or outline of the nucleus. Even the lymphocytes of the blood when observed in wet smears possess a nuclear indentation.[1-7] In the field of inflammation, this lymphocytic indentation is usually noticeable as the cleft becomes deeper in the outstretched cell. Changes in the nuclear membrane are not confined to the region of the widening indentation. Other areas of nuclear membrane at some distance from the indentation show definite undulation suggestive of increased activity at the nuclear cytoplasmic interface. If vacuoles or ingested substances are in contact with the nuclear membrane, they are able to indent the nucleus at the point of contact suggesting again an *increased fluidity of the nucleus. By the 14th hour the nucleus of the lymphocyte* has increased in size. This increase is apparently due to the increased amounts of nuclear juice or parachromatin which latter becomes increasingly distinct (FIGURE 11).

Little is known concerning the functional changes in the lymphocytic nucleus. A beginning in this direction has been made by Thorell[128] who in studying metabolism in the lymphocytes found that the intracellular nucleic acid metabolism of large lymphocytes indicated a high intensity of growth.

Lymphocytic cytoplasm undergoes more apparent structural and functional changes. The most remarkable and earliest of these changes in lymphocytic cytoplasm is the assumption of the phagocytic function (*cf* FIGURES 4 and 9). This ability is possessed by lymphocytes within the blood stream but is usually a latent one. Downey[55, 56] demonstrated that lymphocytes within the blood itself possessed the ability of phagocytosing vital dyes if the dyes were made available to these cells by the simple expedient of double ligature of a vessel followed by study of the lymphocytes in the interposed segment. Included in these phagocytic powers then is the ability to ingest vital dyes (*cf* experiment *H 8*).

Equally rapid in its assumption by the lymphocyte in the area of inflammation is the ability to form a rosette of numerous neutral red vacuoles in the cytoplasm of the nuclear bay. This property of the lymphocytes has been demonstrated by Maximow,[86a] Bloom,[86] Hall,[1, 9, 130] and Cappell.[93] Interestingly enough, the lymphocytes also become peroxidase positive[131] and Nadi positive[132] after a short sojourn in the field of inflammation.

heim and Ferrata [141] with its indented nucleus, functioned quite like the lymphocyte in the event of migration into the site of inflammation However monocytes remained structurally distinct from the lymphocytes and retained their horseshoe-shaped nucleus as they progressively transformed into monocytogenous macrophages monocytogenous giant cells monocytogenous clasmatocytes and finally monocytogenous fibroblasts (FIGURES 19 to 27)

Proponents of a more intensive monocytic origin of macrophages in general submit that the admittedly small number of monocytes available for migration or mobilization at inflammatory sites is offset by the large number of mitoses among the monocytes In our entire series of preparations from lesions in human volunteers mitoses have been absent in the hematogenous exudative cells lymphocytes as well as monocytes

Structural modifications accompanying transformation of blood monocytes from a patient suffering from an intense but maturated leukemic monocytosis into monocytogenous macrophages were gradual loss of the azurophilic granulation characteristic of the monocyte in man increase in cytoplasm particularly hyaloplasm increase in nuclear size in parachromatin and in distinctness of the chromatin network with a striking accentuation of the nuclear indentations (FIGURES 14 to 18)

The role of clasmatocytes resting wandering cells or histogenous macrophages in local inflammation has been established by the schools of Maximow Downey Sabin Doan and Tompkins Our study of responses of these cells in acute inflammation in man adds little that is new to the accepted concepts of clasmatocyte function They were readily phagocytic in the earliest stages of acute inflammation and were the earliest source of the macrophage but because of their limited numbers their inability to migrate great distances and their limited powers of cell division they were of necessity reinforced either by the more mobile monocytes (when available) and/or the more numerous lymphocytes in most lesions

The intact organism in a few strategically situated locations along the lymph and blood streams masses great numbers of histogenous macrophages in order to ingest foreign antigens which make their way into the lymph and blood stream These potential histogenous macrophages are the reticuloendothelial cells and the aggregates which they form are the spleen lymph nodes lymphocytic tissues and bone marrow as well as the sinusoids of several of the endocrine glands The events of inflammation in reticuloendothelial tissues with such massed readily available histogenous macrophages are quite different from those in other regions of the body In such reticuloendothelial organs the histogenous macrophages of course dominate the inflammatory field and hematogenous elements usually play only a secondary role

The great mesothelial lined cavities of the body lie midway in the scale of relative histogenous-hematogenous macrophage participation in the event of inflammation In the exudative responses in these cavities both sources of macrophages are of almost equal importance

It is well to point out that most regions of the body contain relatively fewer histogenous macrophages In the central nervous system they are called microglia in the interstitial connective tissue of the myocardium Anitsck

gradually changed from the polarized "hand mirror" method to that of continuous depolarization characteristic of the monocyte

As observed by the early critics of Metchnikoff and more recently by the Clarks,[115] Hall and Furth[136] and by Florey and his group,[116] some lymphocytes in the field of inflammation or in tissue culture may either fail to hypertrophy or to become phagocytic and retain their coarse, clumped, nuclear pattern or even ultimately degenerate *in situ* Again in areas of chronic inflammation sessile lymphocytes are a common finding Metchnikoff[2] gave the first of several explanations for such findings He held that there is a constant struggle in the field of inflammation between the migrated lymphocytes and the admittedly deleterious environment of the inflammatory lesion, and it is to be expected that some of the lymphocytes will succumb The works of Dougherty and White[137, 138] and Harris Harris and Farber[139, 140] and Wesslen[141] afford another explanation for the presence of sessile lymphocytes Inasmuch as lymphocytes have been demonstrated to be a source of antibodie local aggregates in areas of inflammation may be functioning in the direction of antibody production rather than macrophage transformation Ehrich and his group[11] furthermore, have suggested a trophocytic function for the apparently inactive lymphocytes

In summary, transformation of the lymphocyte into the lymphocytogenous macrophage in acute inflammation is accompanied by the following structural and functional modifications increase in phagocytic ability for cellular debris bacteria and vital dyes increase in nuclear size division of coarse chromatin masses into fine angular pieces increase in parachromatin increase in chromatin parachromatin definition increase in cell body size enlargement of cytocentrum increase in centrioles appearance of astral rays increase in number of neutral red vacuoles with aggregation into a rosettelike apparatus about the cytocentrum assumption of positive peroxidase[131] and Nadi reactions[13] and increasing evidences of depolarization locomotion

The importance of the role of the monocyte in acute inflammation appears to be proportional to the number of monocytes available for emigration or mobilization at the time of the inflammation The morphology of the hypertrophied lymphocytes in FIGURES 2 to 5 and 8 to 11 bears little or no resemblance to that of the monocytes in the insets of the photomicrographs of FIGURES 4 and 5 or in the series represented by FIGURES 14 through 27

The experiments of Murray Webb and Swann,[81] Bloom[84] Frerichs,[118] and those of our third series described above upon local inflammation in animals with high blood monocytoses were in agreement in that only in such animals did the migration of monocytes to the inflammatory site occur in appreciable numbers If the blood of the animals studied contained only the meager normal numbers of monocytes then few were available for migration into the local area of inflammation If the blood of the animals studied contained several thousands of monocytes per cu mm then monocytes migrated in ever increasing numbers into the inflammatory sites In addition our utilization of air-dried preparations of the exudative cells of lesions in experimental animals with concurrent high blood monocytoses permitted direct comparison of migrating monocytes with those of blood smears The large monocyte of Pappen

was followed by the technique described in a patient with monocytic leukemia, Naegeli type in which functionally mature monocytes in the blood outnumbered the lymphocytes Structural modifications accompanying transformation of blood monocytes tagged with prominent nuclear indentations were an increasingly prominent accentuation of the nuclear indentation gradual loss of characteristic dustlike azurophilic granulation, increase in cytoplasm increase in nuclear size in parachromatin and in parachromatin chromatin distinctness The monocytes hypertrophied as an important mobile source of the macrophages in the subject with a concomitant monocytosis in his peripheral blood and key stages of inflammation were hour for hour six μ larger than comparable lymphocytes and their hypertrophied forms

In rabbits with high blood monocytoses brought about by systemic *L monocytogenes* infections a slight modification of the technique employed in man permitted study of the large monocytes with their indented nuclei at the site of inflammation Such monocytes present in great numbers remained structurally distinct from the lymphocytes and their hypertrophied forms in controls and progressively transformed into monocytogenous macrophages monocytogenous giant cell monocytogenous clasmatocytes and monocytogenous fibroblasts

Neutrophilic leukocytes of man migrated at an early stage into the lesions and after performing their functions of phagocytosis and enzyme elaboration, shrank and degenerated Before degeneration they lost their granule-containing cytoplasm to the exudate in the form of small fragments which for a time were free in the inflammatory fluids but which were ultimately ingested by the lymphocytes and hypertrophied lymphocytes as well as by the histogenous macrophages

The literature of leukocytic participation in the inflammatory response was reviewed

Bibliography

1 Dutrochet 1878 Recherches anat et physiol sur la structure interne des anim *etc* (Not listed in the index catalogue of the library of the Surgeon General U S Army Cited by Hektoen & Riesman and Adami[3] *q v*)

2 Hektoen L & D Riesman 1902 An American Textbook of Pathology Esp p 140 Saunders Philadelphia Pa

3 Adami J G 1907 Inflammation 3rd Ed Macmillan London Eng & New York N Y

4 Addison W 1843 The Actual Process of Nutrition in the Living Structure Demonstrated by the Microscope and the Renewal of the Tissues and Secretions with the Phenomena and Products of Inflammation Illustrated and Established 1–76 Churchill London England

5 Waller A 1846 Microscopic observations on the perforation of the capillaries by the corpuscles of the blood and on the origin of mucus and pus globules London Edinburgh and Dublin Philosoph Mag and J Sci 29 397

6 Addison W 1849 On Healthy and Diseased Structure Churchill London England

7 Cohnheim J 1867 Ueber Entzündung und Eiterung Virchows Arch path Anat 40 1 79

8 Zimmerman G 1852 Med Ztg 21 64 (Cited by Schlumberger *q v*)

9 Schlumberger H 1944 Origins of the cell concept in pathology Arch Path 37 396–407

10 Virchow R 1858 Die Cellularpathologie Hirschwald Berlin Germany

11 Schultze M 1865 Ein heizbarer Objecttisch und seine Verwendung bei Untersuchungen des Blutes Arch mikroskop Anat Entwicklungsmech 1 1–42

hows's myocytes", in the lung, septal cells", in the general connective tissues "clasmatocytes or histiocytes, rarely 'adventitial cells' Once the intensity of an inflammatory stimulus exceeds the strength of the normal wear and tear process, neutrophilic leukocytes, lymphocytes and/or monocytes migrate from the vessels reinforcing the histogenous macrophages

Metchnikoff discovered the phagocytic powers of the neutrophilic leukocytes, and Opie[144, 145, 146] did much to help in our understanding of their enzymatic activity Our own preparations obtained in acute inflammation in man and described above have demonstrated an additional subsidiary function of neutrophilic leukocytes Prior to shrinkage and degeneration in the area of inflammation, they lost much of their granule-containing cytoplasm in the form of small fragments which for a time were free in the inflammatory fluids These free portions of neutrophilic cytoplasm were readily ingested by lymphocytes as well as histogenous macrophages In the process of transformation from lymphocyte to macrophage, these ingested fragments may be of service in the elaboration and reorientation of the scant lymphocytic cytoplasm into the abundant cytoplasm of the macrophage

In our preparations the polymorphonuclear basophilic leukocytes of the blood appeared late in inflammation, but were structurally intact Their migration into the field of acute inflammation in man is thus established but further study of their function in such areas awaits the clinical occurrence of high blood basophilias

Finally, because important functions of normal leukocytes are exerted in the tissues application of technical procedures described above to the study of cellular responses in patients affords an additional method for detecting abnormal or distorted leukocytic functions[147, 148, 149]

Summary

By means of an original technical procedure, the cellular exudate of single lesions in man has been sampled hour by hour In 42 lesions cover slip preparations dried and stained like blood smears were obtained which allowed detailed comparison of the exudative leukocytes obtained from the acutely inflamed tissues of man with the leukocytes of blood smears A modification of this technique permitted similar observations of leukocytic functions in the rabbit

When the exciting antigen was one to which the human subject was not systemically immunized transformation of the lymphocytes of man into macrophages was accompanied by the following functional and structural changes in lymphocytes as such increase in cytoplasmic size increase in phagocytic ability for cellular debris and vital dyes assumption of a positive peroxidase reaction assumption of a positive Nadi reaction increase in division of coarse chromatin masses into fine angular pieces increase in nuclear juice or parachromatin increase in irregularity of nuclear membrane and increase in chromatin parachromatin definition

The participation of blood monocytes in local inflammation was in proportion to the number of monocytes available for emigration or mobilization at the time of inflammation In man the role of monocytes in acute inflammation

44 v Verebely T 1907 Die Granulation des menschlichen Fettgewebes Bruns Beitr klin Chir 54 370-349

45 Fischer O 1909 Ueber die Herkunft der Lymphocyten in den ersten Stadien der Entzündung Experimentelle Studie Beitr path Anat u allg m Path 45 400-423

46 Babkina H 1911 Veränderungen der Gewebe der blutbildenden Organe bei aseptischer Entzundung Experimentelle Untersuchun (Inaug D s St Petersburg 1910) Fol ha Haematol 11 207 (Abstract)

47 Homen E A 1911 Studien über experimentelle Tuberkulose in den peripheren Nerven und dem Bindegewebe bei gesunden und bei den alkoholisierten Tieren Arb Path Inst Uni Helsingfors 3 91 138

48 v Bifantt H 1911 Beitrage zur Kenntnis der Pathogenese und Histologie der experimentellen Meningeal und Gehirntuberkul e beim Hunde Arb Path Inst Uni Helsingfors 3 235-601

49 Wallgren A 1911 Beiträge zur Kenntnis der Pathogenese und Histologie der experimentellen Lebertuberkulose Arb Path Inst Univ Helsingfors 3 139-234

50 Downey H & F Weidenreich 1912 Ueber die Bildung der Lymphozyten in Lymphdrusen und Milz Arch mikroskop Anat Entwicklungsmech 80 306-395

51 Dubreuil G 1913 Le chondriome et le dispositif de l'activite ecrétoire Aux différents stades du développement les éléments cellulaires de la lignée connective descendants du lympho-cyte (Globules blancs mononucléées de la lymphe et du sang cellules connectives cartilagineuses et osseuses) Arch Anat micro 15 53 151

52 Aschoff L & K Kiyono 1913 Zur Frage der grossen Mononukleären Fol ha Haematol 15 383

53 Tschaschin S 1913 Ueber die Herkunft und Entstehungsweise der lymphozytoiden (leukozytoiden) Zellen der Polyblasten bei der Entzündung Fol a Haematol 16 247-294

54 Tschaschin S 1914 Ueber die ruhenden Wanderzellen und ihre Beziehungen zu den anderen Zellformen des Bindegewebes und zu den Lymphozyten Fol a Haematol 17 317-397

55 Downey H 1916-17 Histiocytes and macrophages and their relations to the cells of normal blood in animals stained with acid colloidal dyes intravitam Anat Record 11 350-355

56 Downey H 1917 Reactions of blood and tissue cells to acid colloidal dyes under experimental conditions Anat Record 12 429-454

57 Policard A & B Deplas 1917 Les Constituents Cellulaires du tissu de bourgeonnement en evolution normale ou pathologique chez l homme Compt rend mem soc biol 80 745-751

58 Bergel S 1920 Beitrage zur Biologie der Lymphozyten Z exptl Path Therap 21 216-227

59 Dominici H 1920 Etudes sur le tissu conjonctif et les organes hematopoïétiques des mammifères I Tissu conjonctif général II Inflammation du tissu conjonctif III Modifications des organes hematopoïétiques Arch Anat micro 17 3-6 83 136 247-301

60 Latta J 1921 The histogenesis of dense lymphatic tissue of the intestine (Lepus) a contribution to the knowledge of the development of lymphatic tissue and blood cell formation Am J Anat 29 159-211

61 Danchakoff V & B Seidlin 1922 Digestive activity of mesenchyme and its derivatives II Proteins as object (A Edestin) Biol Bull 43 97-122

62 Maximow A 1923 Untersuchungen uber Blut und Bindegewebe VIII Die zytologischen Eigenschaften der Fibroblasten Retikulumzellen und Lymphozyten des lymphoiden Gewebes ausserhalb des Organismus ihre genetischen Wechselbeziehungen und prospektiven Entwicklungspotenzen Arch mikroskop Anat Entwicklungsmech 97 283 313

63 Maximow A 1923 Untersuchungen uber Blut und Bindegewebe X Ueber die Blutbildung bei den Selachiern im erwachsenen und embryonalen Zustande Arch mikroskop Anat Entwicklungsmech 97 623 71

64 Maximow A 1924 Tuberculosis of mammalian tissue *in vitro* J Infectious Diseases 34 549-584

65 Alfejew S 1924 Die embryonal Histogenese der Zellformen des lockeren Bindegewebes der Saugetiere Fol a Haematol 30 111 172

66 Maximow A 19 Ueber krebsahnliche Verwandlung der Milchdruse in Gewebskulturen Virchows Arch path Anat 256 813 845

67 Timofejewsky A & S Benewolenskaja 1925 Explantationsversuche von weissen Blutkorperchen mit Tuberkelbazillen Arch exptl Zellforsch Gewebezucht 2 31 42

12 HAECKEL E H 1862 Die Radiolarien Reimer Berlin Germany
13 SCHULTZE, F E 1866 Objectträger zur Beobachtung lebender Froschlarven Arch mikroskop Anat Entwicklungsmech 2 378
14 HERING, F 1867 Zur Lehre vom Leben der Blutzellen I Ueberwanderung der Blutzellen aus den Blutgefassen in die Lymphgefasse Sitzber Akad Wiss Wien Math naturw Klasse Abt II 56 691
15 LIEBERKUHN N 1870 Ueber Bewegungserscheinungen der Zellen 357-365 N G Elwert III Ed Die farblosen Blutkorper Marburg & Leipzig Germany
16 BIZZOZERO 1871 Gazz med lombarda 1872 Wien med Jahresber 160 (Both cited by Metchnikoff q v)
17 ZIEGLER E 1875 Experimentelle Untersuchungen über die Herkunft der Tuberkelelemente Staudinger Würzburg Germany
18 ZIEGLER F 1876 Ueber die pathologische Gewebe und Gefassneubildung Wurzburg Germany
19 GAY F P 1935 The cellular basis of natural resistance Ch 16 *In* Agents of Disease and Host Resistance Thomas Springfield Ill
20 METCHNIKOFF E 1883 (August) Ueber die Heilkrafte des Organismus In einer allgemeinen Sitzung der Versammlung russischer Naturforscher und Aerzte in Odessa
21 METCHNIKOFF E 1883 Untersuchungen über die mesodermalen Phagocyten einiger Wirbeltiere Biol Centr 3 560
22 METCHNIKOFF E 1893 Lectures on the Comparative Pathology of Inflammation (1891) Trans by F & E Starling Kegan Paul Trench, Trubner & Co London England
23 METCHNIKOFF E 1905 Immunity in Infective Disease 78 Trans by F G Binnie Univ Press Cambridge England
24 LEBER T 1888 Ueber die Entstehung der Entzündung und die Wirkung der entzundungserregenden Schadlichkeiten Fortschr Med 6 460-464
25 METCHNIKOFF E 1888 Ueber die phagocytäre Rolle der Tuberkelriesenzellen Arch path Anat Physiol (Virchow s) 113 63-94
26 YERSIN A 1888 Etude sur le développement du tubercule experimental Ann inst Pasteur 2 246-266
27 RUFFER A 1890 The phagocytes of the alimentary canal Quart J Microscop Sci 30 481-505
28 ARNOLD J 1893 Altes und Neues uber Wanderzellen Arch path Anat Physiol (Virchow s) 132 502-505
29 BORREL A 1894 Tuberculose experimentale du rein Ann inst Pasteur 8 65-87
30 KANTHACK A & W HARDY 1894-95 Morphology and distribution of the wandering cells of mammalia J Physiol 17 81-119
31 RANVIER L 1900 Des Clasmatocytes Arch Anat micro 3 123-139
32 MAXIMOW A 1902 Experimentelle Untersuchungen uber die entzundliche Neubildung von Bindegewebe Beitr path Anat u allgem Path Suppl 5 1 262
33 MAXIMOW A 1903 Weiteres uber Entstehung Struktur und Veranderungen des Narbengewebes Beitr path Anat u allgem Path 34 153-188
34 MAXIMOW A 1904 Ueber entzundliche Bindegewebeneubildung bei der weissen Ratte und die dabei auftretenden Veränderungen der Mastzellen und Fettzellen Beitr path Anat u allgem Path 35 93-126
35 ZIEGLER K 1904 Histologische Untersuchungen uber das Oedem der Haut und das Unterhautzellgewebes Beitr path Anat u allgem Path 36 435-505
36 SCHWARTZ C 1904 Ueber die Herkunft der einkernigen Exsudatzellen bei Entzundungen Wien klin Wochschr 17 1173-1175
37 HELLY K 1905 Zur Morphologie der Exsudatzellen und zur Spezifitat der weissen Blutkörperchen Beitr path Anat u allgem Path 37 171-278
38 MAXIMOW A 1905 Beitrage zur Histologie der eiterigen Entzundung Beitr path Anat u allgem Path 38 301-353
39 LEWIS W H & L T WEBSTER 1921 Migration of lymphocytes in plasma cultures of human lymph nodes J Exptl Med 33 261-270
40 MAXIMOW A 1906 Ueber entzundliche Bindegewebsbildung beim Axolotl Beitr path Anat u allgem Path 39 30-313
41 ZIELER K 1907 Ueber Exsudatzellen bei der aseptischen Entzundung des Bindegewebes Zentr allgem Path u path Anat 18 289-294
42 ZIELER K 1907 Ueber die bei der aseptischen Entzündung des Bindegewebes auftretenden Zellformen Arch Dermatol u Syphilis 86 323-360
43 RENAUT J 1907 Les cellules connectives rhagiocrines Arch Anat micro 9 495-606

44 v Verebely T 1907 Die Granulation des menschlichen Fettgewebes Bruns Beitr klin Chir 54 370–349
45 Fischer O 1909 Ueber die Herkunft der Lymphocyten in den ersten Stadien der Entzündung Experimentell Studie Beitr path Anat u allgem Path 45 400–473
46 Babkina H 1911 Veränderungen der Gewebe der blutbildenden Organe bei aseptischer Entzundung Experimentelle Untersuchung (Inaug Diss St Petersburg 1910) Fol ha Haematol 11 207 (Abstract)
47 Homén F A 1911 Studien über experimentelle Tuberkulose in den peripheren Nerven und dem Bindgewebe bei gesunden und bei den alkoholisierten Tieren Arb Path Inst Univ Helsingfors 3 31 138
48 v Fieandt H 1911 Beitrage zur Kenntnis der Pathogenese und Histologie der experimentellen Meningeal und Gehirntuberkulose beim Hunde Arb Path Inst Univ Helsingfors 3 235–601
49 Wallgren A 1911 Beiträge zur Kenntnis der Pathogenese und Histologie der experimentellen Lebertuberkulose Arb Path Inst Univ Helsingfors 3 139–234
50 Downey H & F Weidenreich 1912 Ueber die Bildung der Lymphozyten in Lymphdrusen und Milz Arch mikrosk Anat Entwicklungsmech 80 306–395
51 Dubreuil, C 1913 Le chondriome et le dispositif de l'activé sécrétoire Aux différents stades du développement des éléments cellulaires de la lignée connective descendants du Lymphocyte (Globules blancs mononucléés de la lymphe et du sang cellules connectives cartilagineuses et osseuses) Arch Anat micro 15 53 151
52 Aschoff L & K Kiyono 1913 Zur Frage der grossen Mononukleären Folia Haematol 15 383
53 Tschaschin S 1913 Ueber die Herkunft und Entstehungsweise der lymphozytoiden (leukozytoiden) Zellen der Polyblasten bei der Entzündung Folia Haematol 16 24 –294
54 Tschaschin S 1914 Ueber die ruhenden Wanderzellen und ihre Beziehungen zu den anderen Zellformen des Bindegewebes und zu den Lymphozyten Folia Haematol 17 31 377
55 Downey H 1916–17 Histiocytes and macrophages and their relations to the cells of normal blood in animals stained with acid colloidal dyes intravitam Anat Record 11 350–355
56 Downey H 191 Reactions of blood and tissue cells to acid colloidal dyes under experimental conditions Anat Record 12 42)–454
57 Policard A & B Desplas 1917 Les Constitue ts Cellulaires du tissu de bourgeonnement en évolution normale ou pathologique chez l homme C mpt rend mem soc biol 80 745–751
58 Bergel S 1920 Beitrage zur Biologie der Lymphozyten Z exptl Path Therap 21 216–227
59 Dominici H 1920 Etudes sur le tissu conjonctif et les organes hematopoïétiques des mammifères I T u conjonctif général II Inflammation du tissu conjonctif III Modifications des organes hematopoïétiques Arch Anat micro 17 3– 6 83–136 24 –301
60 Latta J 1921 The histogenesis of dense lymphatic tissue of the intestine (Lepus) a contribution to the knowledge of the development of lymphatic tissue and blood cell formation Am J Anat 29 159–211
61 Danchakoff V & B Seidlin 1922 Digestive activity of mesenchyme and its derivatives II Proteins as object (A Edestin) Biol Bull 43 9 –122
62 Maximow A 1923 Untersuchungen über Blut und Bindegewebe VIII Die zytologischen Eigenschaften der Fibroblasten Retikulumzellen und Lymphozyten des lymphoiden Gewebes ausserhalb des Organismus ihre genetischen Wechselbeziehungen und prospektiven Entwicklungspotenzen Arch mikroskop Anat Entwicklungsmech 97 283 313
63 Maximow A 1923 Untersuchungen über Blut und Bindegewebe X Ueber die Blutbildung bei den Selachiern im erwachsenen und embryonalen Zustande Arch mikrosk p Anat Entwicklungsmech 97 623– 1
64 Maximow A 1924 Tuberculosis of mammalian tissue in vitro J Infect ous Diseases 34 549–584
65 Alfejew S 1924 Die embryonale Histogenese der Zellformen des lockeren Bindegewebes der Saugetiere Fol Haemat 1 30 111 172
66 Maximow A 1925 Ueber krebsahnliche Verwandlung der Milchdrüse in Gewebskulture Virchows Arch path Anat 256 813 84
67 Timofejewsky A & S Benewolenskaja 1925 Explantationsversuche on weissen Blutkorperchen mit Tuberkelbazillen Arch exptl Zellforsch Gewebezücht 2 31–47

12 Haeckel E H 1862 Die Radiolarien Reimer Berlin Germany
13 Schultze F E 1866 Objectträger zur Beobachtung lebender Froschlarven Arch mikroskop Anat Entwicklungsmech 2 378
14 Hering E 1867 Zur Lehre vom Leben der Blutzellen I Ueberwanderung der Blutzellen aus den Blutgefassen in die Lymphgefasse Sitzber Akad Wiss Wien Math naturw Klasse Abt II 56 691
15 Lieberkühn N 1870 Ueber Bewegungserscheinungen der Zellen 357-365 N G Elwert III Ed Die farblosen Blutkörper Marburg & Leipzig Germany
16 Bizzozero 1871 Gazz med lombarda 1872 Wien med Jahresber 160 (Both cited by Metchnikoff q v)
17 Ziegler E 1875 Experimentelle Untersuchungen über die Herkunft der Tuberkelelemente Staudinger Würzburg Germany
18 Ziegler E 1876 Ueber die pathologische Gewebe und Gefassneubildung Würzburg Germany
19 Gay F P 1935 The cellular basis of natural resistance Ch 16 *In* Agents of Disease and Host Resistance Thomas Springfield Ill
20 Metchnikoff E 1883 (August) Ueber die Heilkrafte des Organismus In einer allgemeinen Sitzung der Versammlung russischer Naturforscher und Aerzte in Odessa
21 Metchnikoff E 1883 Untersuchungen über die mesodermalen Phagocyten einiger Wirbeltiere Biol Centr 3 560
22 Metchnikoff E 1893 Lectures on the Comparative Pathology of Inflammation (1891) Trans by F & E Starling Kegan Paul Trench Trübner & Co London England
23 Metchnikoff E 1905 Immunity in Infective Disease 78 Trans by F G Binnie Univ Press Cambridge England
24 Leber T 1888 Ueber die Entstehung der Entzündung und die Wirkung der entzündungserregenden Schädlichkeiten Fortschr Med 6 460-464
25 Metchnikoff E 1888 Ueber die phagocytare Rolle der Tuberkelriesenzellen Arch path Anat Physiol (Virchow s) 113 63-94
26 Yersin A 1888 Etude sur le développement du tubercule experimental Ann inst Pasteur 2 246-266
27 Ruffer A 1890 The phagocytes of the alimentary canal Quart J Microscop Sci 30 481-505
28 Arnold J 1893 Altes und Neues uber Wanderzellen Arch path Anat Physiol (Virchow s) 132 502-505
29 Borrel A 1894 Tuberculose experimentale du rein Ann inst Pasteur 8 65-82
30 Kanthack A & W Hardy 1894-95 Morphology and distribution of the wandering cells of mammalia J Physiol 17 81-119
31 Ranvier L 1900 Des Clasmatocytes Arch Anat micro 3 123-139
32 Maximow A 1902 Experimentelle Untersuchungen über die entzündliche Neubildung von Bindegewebe Beitr path Anat u allgem Path Suppl 5 1-262
33 Maximow A 1903 Weiteres uber Entstehung Struktur und Veränderungen des Narbengewebes Beitr path Anat u allgem Path 34 153-188
34 Maximow A 1904 Ueber entzündliche Bindegewebeneubildung bei der weissen Ratte und die dabei auftretenden Veranderungen der Mastzellen und Fettzellen Beitr path Anat u allgem Path 35 93-126
35 Ziegler K 1904 Histologische Untersuchungen über das Oedem der Haut und das Unterhautzellgewebes Beitr path Anat u allgem Path 36 435-505
36 Schwartz G 1904 Ueber die Herkunft der einkernigen Exsudatzellen bei Entzündungen Wien klin Wochschr 17 1173-1175
37 Helly K 1905 Zur Morphologie der Exsudatzellen und zur Spezifitat der weissen Blutkörperchen Beitr path Anat u allgem Path 37 171-278
38 Maximow A 1905 Beitrage zur Histologie der eiterigen Entzundung Beitr path Anat u allgem Path 38 301-353
39 Lewis W H & I T Webster 1921 Migration of lymphocytes in plasma cultures of human lymph nodes J Exptl Med 33 261-270
40 Maximow A 1906 Ueber entzundliche Bindegewebsbildung beim Axolotl Beitr path Anat u allgem Path 39 30-313
41 Zieler K 1907 Ueber Exsudatzellen bei der aseptischen Entzundung des Bindegewebes Zentr allgem Path u path Anat 18 289-294
42 Zieler K 190 Ueber die bei der aseptischen Entzundung des Bindegewebes auftretenden Zellformen Arch Dermatol u Syphilis 85 323-360
43 Renaut J 1907 Les cellules connectives rhagiocrines Arch Anat micro 9 495-606

92 Stieve H 1929 Das Mesenchym in der Wand der menschlichen Gebärmutter Zentr Gynäkol 53 2 06-2 23
93 Higgins G & B Palmer 1929 The origin of fibroblasts within an experimental hematoma Arch Path 7 63-70
94 Kreyberg L 1929 The prospective potencies of the human lymphocyte Arch exptl Zellforsch Gewebezücht 8 359 364
95 Timofejewsky A & S Benewolenskaja 1929 Neue Beobachtungen an lymphoiden Zellen der myeloiden und lymphatischen Leukämie in Explantations-Versuchen Arch exptl Zellforsch Gewebezücht 8 1 34
96 Silberberg M 1930 Lymphocyten und Histiocyten Klin Wochschr 9 174-1 6
97 Sabin F P C A Doan & C E Forkner 1930 Reaction of the connective tissues of the normal rabbit to lipoids from the tubercle bacillus strain H 37 J Exptl Med Suppl 3 52 1-88
98 Cappell D F 1930 Intravitam and supravital staining IV The cellular reactions following mild irritation of the peritoneum in normal and vitally stained animals with special reference to the origin and nature of the mononuclear cells J Path Bact 33 429-452
99 Erola M 1931 Reactions of subcutaneous tissue to sodium ricinoleate and other foreign substances Folia Haematol 43 454-474
100 Pierce M 1932 Cultures of leukemic blood leukocytes Arch Path 14 295-322
101 Seki M 1935 Zur Kenntnis der intra und supravitalen Färbung IX Z Zellforsch u mikroskop Anat 23 314-331
102 Taliaferro W & L Cannon 1936 The cellular reactions during primary infections and superinfections of Plasmodium brasilianum in Panamanian monkeys J Infectious Diseases 59 72 125
103 Taliaferro W & H Mulligan 1937 The histopathology of malaria with special reference to the function and origin of the macrophages in defense Indian Med Research Mem 29 1 138
104 Hertzog A 1938 The phagocytic activity of human leukocytes with special reference to their type and maturity Am J Path 14 595-605
105 Kolouch F 1939 The lymphocyte in acute inflammation Am J Path 15 413-433
106 Taliaferro W & C Kluver 1940 The hematology of malaria (Plasmodium brasilianum) in Panamanian monkeys 2 Morphology of leukocytes and origin of monocytes and macrophages J Infectious Diseases 67 162-176
107 Finlayson A & J Latta 1940 The early reaction in the leptomeninges of the rabbit to trypan blue Anat Record 78 281 291
108 Plimpton N 1940 Basophil leukocytes and myelocytes after local injection of ventriculin Anat Record 78 475-484
109 Tompkins E H 1940 A comparison between the reactions of the large phagocytic mononuclear cells to subcutaneous injection of individual lipids and to mixtures of the same lipids Southern Med J 33 154 159
110 Tompkins E H 1946 Reaction of the reticuloendothelial cells to subcutaneous injections of cholesterol Experimental animals mice Arch Path 42 299-319
111 Tompkins F H 1953 Resting reticuloendothelial cells and their fluid accuoles reactions to potassium tonic t and albumin Bull Johns Hopkins Hosp 92 9-93
112 Tompkins E H & M A Cirillo 1935 Factors favoring phagocytosis by reticulo-endothelial cells early in inflammation Am J Path 29 21 232
113 Gray M F 1940 A supravital study of the cells of subcutaneous tissue in guinea pigs following injections of synthetic triglycerides Am J Anat 67 361-392
114 Berman L 1942 Observations on dry films of cultures of lymphoid tissues Arch Path 38 295-304
115 Clark E R F L Clark & R O Rex 1936 Observations on polymorphonuclear leukocytes in the living animal Am J Anat 59 123-1 3
116 Ebert R H A C Sanders & H W Florey 1940 Observations on lymphocytes in chambers in the rabbit's ear Brit J Exptl Path 21 212 218
117 Harper S 194 Fibrous wound healing as seen in transparent chambers inserted in the ears of rabbit Thesis Univ of Minnesota (Mayo Clinic)
118 Frerichs J B 1944 The influence of a monocytosis of the peripheral blood stream upon the cellular character of an acute inflammation Bull John Hopkins Hosp 74 45-54
119 Rey H 1945 Cellular reactions in the dermal connective tissue of the hamster to *Leishmania brasiliensis* J Infectious Diseases 72 117 124

68 JORDAN H 1925 A study of the blood of the leopard frog by the method of supra vital staining combined with injection of India ink into the dorsal lymph sac with special reference to the genetic relationships among leucocytes Am J Anat 35 105–132
69 STILLWELL F 1926 On the phagocytic capacity of the blood vessel endothelium of the frog s tongue and its presumed transformation into wandering cells Folia Haematol 33 81–94
70 VIERLING A 1926 Experimenteller Beitrag zur Geschichte der Wanderzellen bei Amphibien Z Anat Entwicklungsgeschichte 81 448–464
71 LANG F 1926 Role of endothelium in the production of polyblasts (mononuclear wandering cells) in inflammation Arch Path 1 41–63
72 TIMOFEJEWSKY A & S BENEWOLENSKAJA 1927 Tuberculous inoculations in the cultures of leukocytes of the human blood Arch exptl Zellforsch Gewebezucht 4 64–78
73 MICHELS N & J GLOBUS 1927 The so-called small round cell infiltrations I Polio encephalitis and acute epidemic encephalitis Arch Path 4 692–731
74 WATSON C J 1928 The pathology of histoplasmosis (Darling) with special reference to the origin of phagocytic cells Folia Haematol 37 70–93
74a DOWNEY H 1915 The so called endothelioid cells Anat Record 9 73–77
74b DOWNEY H 1918–19 Further studies on the reactions of blood and tissue cells to acid colloidal dyes Anat Record 15 103–134
75 MAXIMOW A 1927 Bindegewebe und blutbildende Gewebe Handbuch der microscropischen Anatomie des Menschen 2(Part 1) W v Mollendorf Ed Die Gewebe Berlin Germany
76 LEWIS W H 1925–26 The transformation of mononuclear blood cells into macro phages epithelioid cells and giant cells Harvey Lectures 21 77–112
77 CARREL A & A H EBELING 1926 Transformation of monocytes into fibroblasts through the action of Rous virus J Exptl Med 43 461–468
78 SIMPSON M I 1922 The experimental production of macrophages in the circulating blood J Med Research 43 77–144
79 SABIN F R C A DOAN & R S CUNNINGHAM 1925 Discrimination of two types of cells in the connective tissue by the supravital technique Carnegie Inst Technol Contribs Embryol No 82 16 125
80 CUNNINGHAM R S F R SABIN S SUGIYAMA & J A KINDWALL 1925 The role of the monocyte in tuberculosis Bull Johns Hopkins Hosp 37 231–280
81 MURRAY F G D R A WEBB & B I SWANN 1926 A disease of rabbits characterized by a large mononuclear leukocytosis caused by a hitherto undescribed bacillus *Bacterium monocytogenes* (n p) J Path Bact 29 407–439
82 WITTS L J & R A WEBB 1927 The monocytes of the rabbit in *B monocytogenes* infection a study of their histogenesis and staining reactions J Path Bact 30 687–712
83 BLOOM W 1928 Origin and nature of the monocyte Folia Haematol 37 1–62
84 BLOOM W 1928 The formation of abscesses in an infection with *Bacterium monocytogenes* Arch Path 6 995–1007
85 BLOOM W 1928 The relationship between lymphocytes monocytes and plasma cells Folia Haematol 37 63 69
86 BLOOM W 1928 Mammalian lymph in tissue culture From lymphocyte to fibroblast Arch exptl Zellforsch Gewebezucht 5 269–307
86a MAXIMOW A 1928 Cultures of blood leukocytes From lymphocyte and monocyte to connective tissue Arch exptl Zellforsch Gewebezucht 5 169–268
87 CAFFIER P 1928 Die prospektiven Potenzen des normalen Menschenblutes Arch exptl Zellforsch Gewebezucht 6 285–296
88 TIMOFEJEWSKY A & S BENEWOLENSKAJA 1928 Züchtung von Geweben und Leukocyten des Menschen mit Tuberkelbacillen Calmettes (BCG) Virchow s Arch path Anat 268 829–651
89 LACASSAGNE A & GRICOUROFF 1928 De l action des radiations sur les leucocytes du sang étudiée au moyen de la méthode des cultures Arch exptl Zellforsch Gewebezucht 6 303 315
90 MICHELS N & J GLOBUS 1929 The so called round cell infiltrations II Syphilis of the central nervous system Arch Path 8 371 418
91 STIEVE H 1929 Muskulatur und Bindegewebe in der Wand der menschlichen Gebärmutter ausserhalb und während der Schwangerschaft während der Geburt und des Wochenbettes Akad Verlagsgesellschaft Leipzig Germany

PLATES

120 DOUGHERTY T F 1944 Studies on the cytogenesis of microglia and their relation to cells of the reticulo-endothelial system Am J Anat 74 61–96

121 DE BRUYN P 1945 The motion of the migrating cells in tissue cultures of lymph nodes Anat Record 93 295–306

122 CAMPBELL B & R A GOOD 1949 Antigen antibody mechanisms in neutrotropic virus diseases Ann Allergy 7 471–481

123 TOWNSEND W A & B CAMPBELL 1949 The effects of roentgen rays on the inflammatory cells of the mouse and rabbit Blood 4 1346–1356

124 GOOD R A 1950 Experimental allergic brain inflammation a morphological study J Neuropathol Exptl Neurol 9 78–92

125 DOUGHERTY T F & G L SCHNEEBELI 1950 Role of cortisone in regulation of inflammation Proc Soc Exptl Biol Med 75 854–859

126 DOWNEY H 1938 Monocytic leucemia and leucemic reticulo-endotheliosis Section XVI in Handbook of Hematology 2 1273–1334 H Downey Ed Hoeber New York N Y

127 MAXIMOW A 1928 The lymphocytes and plasma cells *In* Special Cytology 322 E V Cowdry Ed Hoeber New York N Y

128 THORELL B 1945 Om nukleotidomsattningen i lymfocyterna Nord Med 28 2115–2116

129 HALL B E 1930 A critical review of the hematological literature dealing with the results of the supravital staining method Folia Haematol 43 206

130 HALL B F 1938 Evaluation of the supravital staining method *In* Handbook of Hematology 641–698 H Downey Ed Hoeber New York N Y

131 REBUCK J W & F A MONAGHAN 1948 Peroxidase in the lymphocytes of man in acute inflammation Federation Proc 7 277–278

132 REBUCK J W 1952 Enhanced Nadi reaction in lymphocytes in inflammation Federation Proc 11 424–425

133 JONES O P 1947 Mitochondria and their relation to the so-called hyaloplasm J Lab Clin Med 32 700–719

134 GOMORI G 1952 Microscopic Histochemistry 273 Univ Chicago Press Chicago Ill

135 WISLOCKI G B & E W DEMPSEY 1946 Observations on the chemical cytology of normal blood and hemopoietic tissues Anat Record 96 249–278

136 HALL J W & J FURTH 1938 Cultural studies on the relationship of lymphocytes to monocytes and fibroblasts Arch Path 25 46–59

137 DOUGHERTY T F J H CHASE & A WHITE 1944 The demonstration of antibodies in lymphocytes Proc Soc Exptl Biol Med 57 295–298

138 DOUGHERTY T F A WHITE & J H CHASE 1945 Relationship of antibody content of normal and malignant lymphocytes Proc Soc Exptl Biol Med 59 172–175

139 HARRIS T N S HARRIS & M B FARBER 1954 Studies on the transfer of lymph node cells I Appearance of antibody in recipients of cells from donor rabbits injected with antigen J Immunol 72 148–160

140 HARRIS T N S HARRIS & M B FARBER 1954 Studies on the transfer of lymph node cells II Effects of experimental manipulation of the donor system J Immunol 72 161–171

141 WESSLEN T 1952 Studies on the role of lymphocytes in antibody production Acta Dermato-Venereol 32 265–273

142 WAGNER B M & W I EHRICH 1950 Adenosinase adenase and xanthine oxidase of lymphoid tissues Federation Proc 9 347–348

143 PAPPENHEIM A & A FERRATA 1910 Ueber die verschiedenen lymphoiden Zellformen des normalen u pathologischen Blutes Folia Haematol 10 78

144 OPIE E L 1905 Enzymes and anti enzymes of inflammatory exudates J Exptl Med 7 316–334

145 OPIE E L 1906 The enzymes in phagocytic cells of inflammatory exudates J Exptl Med 8 410–436

146 OPIE E L 1922 Intracellular [illegible] Physiol Revs 2 552–58

147 REBUCK J W R W SMITH & K R MARGULIS 1951 The [illegible] leukocytic functions in human [illegible] Gastroenterology 19 [illegible]

148 REBUCK J W & K C MELLINGER 1953 Interruption by topical [illegible] of leukocytic cycles in acute inflammation in man Ann N Y Acad Sc 56 [illegible]

149 REBUCK J W & J L YATES 1954 The cytology of the tuberculin reaction in skin windows in man Am Rev Tuber 69 216–226

PLATE 1

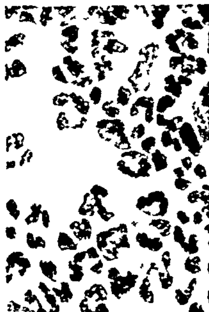
FIGURE 1 A lymphocyt d m u n t ph l th l i n of a norm l m le (H 8) i the th d ho of i fl mmat i ma × 2000 (ed d ne half) AFIP N g 94132

FIGURE 2 Fo lymphocyt s a d h me s n t ph l i th sam l n s FIGURE 1 n th th h r f nfl mm t o i m × 2000 (ed d h lf) AFIP N g 93770

FIGURE 3 E ght en lymph yte n tr ph l sam le n a FIGURE 1 (H-8) n the n th h u of fl mm t ma × 2000 (du d h lf) AFIP N g 96496

FIGURE 4 N lymphocyt s d t ph l n th sam l s s FIGURE 1 i the 12th h f fl m m t n ma l set m cyt f m th blood f th sam i d d l to be c mp ed w th lymph cyt to s d stru t al char ct t B th × 2000 (du d h lf) AFIP N g 93771 a d 95230









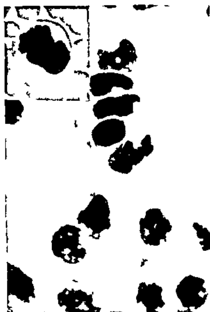
PLA E 2

F U E 5 Lymph yte a d hypert oph d lymphocyt nd a s gl ryk ot eut ph l n the same l n s FIGU E 1 the 14th h r f fl mm ti n n man l s t a m yt fr m th blood f th m d f al to b omp r d w th th lymph yte a t a d st uctur l cha acte t B th × 2000 (red d h lf) AFIP Neg 96495 nd 95 30

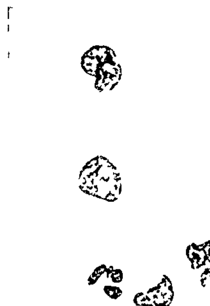
FIGU E 6 Lymph ytog n u m ophage i the m l as FI U E 1 (H 8) i th 21st hour f flamm t n n man × 2000 (d d o h lf) AFIP Neg 9 231

FIGU 7 Th e m r ph ge a d t ph l i the sam l o a FIGURE 1 (H 8) in th 24th ho of i fl mmat on i m × 2000 (r d d o h lf) AFIP N g 96491

FIGURE 8 Tw lymphocyte d m o s ne t ph l th l o of a norm l mal (H 11) n th f fth h u of inflam n t n a m × 2000 (r d d e half) AFIP N g 935 9







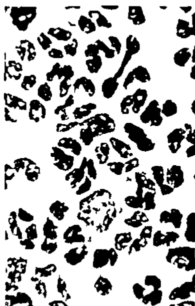


PLATE 2

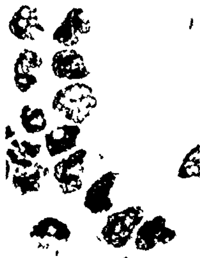
FIGURE 5 Lymph yt a d bypert ph d lymphocyt a d a gle pyknot n ut ph l the s me l a FIGU E 1 th 14th h f flammat n n m n I t a m n cyt f m th l lood f th s m nd d l t b mp d w th the lymph cyt s t s e nd tru t l ch act st B th × 2000 (d c d n h lf) AFIP N g 96495 a d 95 30

FIGURE 6 Lymphocyt g n ma phag th m les n as FIGURE 1 (H-8) in th 21 t h r f fl mmat n m × 2000 (r d ced o half) AFIP N g 9 231

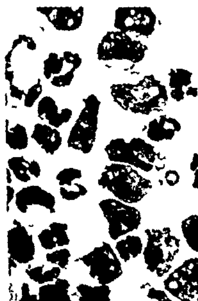
FIGURE 7 Th m c ph ges d t oph l th am l s FIGURE 1 (H 8) n the 24th h f fl mm t n n m n × 2000 (r d c d o h lf) AFIP N g 96491

FIGURE 8 Two lymphocyt s nd um n ut ph l th l n of a n mal mal (H 11) th fifth h of nflamm t m n × 2000 (d d o half) AFIP N g 93 39





10



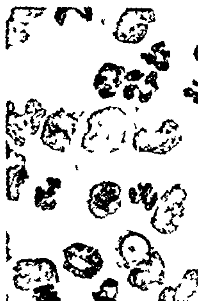


PLATE 3

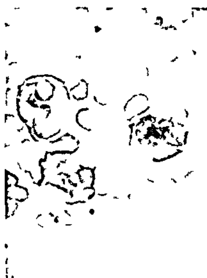
FIGURE 9 Te lymphocyt s se al of them ph gocytic d e hrunken n ut oph l n th same l s n a FIGURE 8 (H 11) in th 12th h r f nfl mm ti n m × 2000 (r du ed h lf) AFIP N g 93 01

FIGURE 10 Se en lymphocyte fo hypert oph d lymphocytes d t o d gen r t g n troph ls the same les o as FIGURE 8 (H 11) at the 1 th ho f fl mm t m n × 2000 (educ d o h lf) AFIP N g 93503

FIGURE 11 Tw l e hype t ph d lymph yt d 13 d g e at ng neut ph ls the sam le a FIGURE 8 (H 11) t th 14th h f fl mmat n ma × 2000 (r d ed o h lf) AFIP Neg 93560

FIGURE 12 T lymphocyt se n lymphocyt ge ma ph ge and deg ner t g tr ph l th sam l o s FIGURE 8 (H 11) afte 16 5 ho r of t fl mm t n n m × 2000 (d d e half) AFIP N g 93508









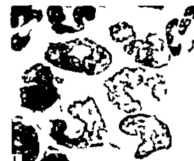




PLATE 4

FIGURE 13 F ur mac ph ge and a po ti f a fifth n th s m le ion as FIGURE 8 (H 11) at th 28th hour of i flammat on in m × 2000 (ed d e half) AFIP Neg 93558

FIGU E 14 Th mo cytes n the blood f a p t nt uffer g f om N eg l type mo ocyt c l ukem a Mo tha 6000 s b cells w re f nd per u mm × 1800 (r duced n half)

FIGURE 15 A m ocyt n the l s n (L 1) f th pat nt w th mo ocyt c leukem a d p ct d r GURE 14 at *th f u th b r of nfl mmatio n ma × 1800 (r d ced on h lf)*

FIGURE 16 F ur mon cyt a d two ne t ph l th same les n FIGU E 15 (L-1) of th p t e t with mo cyt c l kem at the sev th h ur f flammat i m × 1800 (d d on half)

F GURE 17 F e hype t oph d mo ocyt n the m l n as F GURE 15 (L 1) of th p t e t w th mon cytic leukem a at th 12th h ur f fl mm t n i m × 1800 (d d o h lf)

FIGURE 18 T o m o yt g n u m c phag s n th am l o a FI UR 15 (L 1) of t p t t w th mo ocyt le kem t 12 5 h u s f fl mmat Th ell FI UR S 14 18 ho ld b c mp l w th th c ntr l lls in FIGURES 1 13 × 1800 (r du d e half)









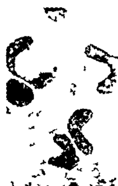










PLATE 5

FIGU E 19 Two mo ocyt a d h te oph l th blood f a abb t (L-40) r prese tati f th pe km o-yt s h d (7 300 m nocyt pe mm) 60 h ur fter t n s dm n t ti f L m yt g × 1800 (red d o h lf)

FIGURE 20 A m ocyte a d a d t g at g hete ph l th l on of rabb t L-40 (th per ph al blood monocyt s a d p ct d n FI URE 19) at 22 h s f nfl mm ti n × 1800 (r d d e half)

F RE 21 Thr mon yt ge o ma ophages and tw ma phage n t f mo ocyti g n th s m l f abbit L-40 d p t d F G R 20 t 31 5 h u s f i flammat × 1800 (d ed o h lf)

FIGU E 22 A m ocyte a d tw m cyt g o s m r j h g s d se e l d t grat g h t ph l the sam le f abb t L-40 (w th per ph l blood m nocyt d p ted in FIGURE 19) at 41 h f i flam t n × 1800 (d c d o e h lf)

FIGUR 23 F m ocytoge m rophage d m cr ph ge ot f m ocyt o g th sam le f r bbit L-40 d p t d FIGU E 20 (w th pe ph l bl od mo cyt s dep t d i F GU E 19) at 46 h s f i fl mmat n × 1800 (d ed o h lf)

FIGUR 24 Hyb d g nt ell w th th n m nocyt c mpo e t a d m ocyt c ompo t t th sam l s f r bb t L-40 d p t d n IGUR 2) (w th pe ph al bl od m cyto s d p t d i FI U E 19) t 57 h ur of fl mm t n × 1800 (ed d o e h lf)

F U E 25 Hyb d g t ll w th mo ocyt a d m ocyt mpo t the same l f bb t L-40 d p t d i F GU E 20 (w th p phe l blo d m ocyt s d p ct d i FIGU E 19) t 0 h u f nflamma t × 1800 (ed ed on h lf)

FIGUR 26 S l m ocyt g o ma ph g w la m tocyt i the sam l i n f r bb t L-40 de p t d n F GURE 0 (w th p p h l blood m ocyt d p t d F GURE 19) at 70 ho s of nfl mm t n × 1800 (d ed h lf)

F GUR 27 Cl m tocyt f b bl t f m l m ytog n m ph ge i th am l o f bb t L-40 dep t d RE 20 (w th the pe ph al blood m ocyt d p t d n FIGU E 19) t 0 h rs f fl mma t × 1800 (r d d ne h lf)

and the method of Tullis[27] of direct perfusion of nutrient fluid through one end of a segment of human rib have also to date been relatively unsuccessful in giving absolute increases in cell number or long term growth of human leukocytes

All these methods however may in the future be adapted to successful cultures when the principles to be described which were derived from the gradient culture are applied to them

The suspension type culture in a vaccine vial capped bottle[28] developed in the Division of Experimental Medicine at the University of Oregon Medical School has been until recently the most satisfactory method for quantitative controlled studies on human marrow or leukemic blood A few of the applications of tissue-culture methods to the study of human leukocytes may be illustrated by studies made by this method Any variable which may be studied in blood may be studied in these cultures and by the same methods All manipulations are with syringe and needle through 70 per cent alcohol on the vaccine caps

Studies of the action of various antibiotics on a known number of a known type of organism in such cultures provided much information on phagocytosis[6,7] and showed that cells immature enough to be in the process of mitosis could still phagocytose bacteria (see Figure 4 in reference 28) Such studies also showed that phagocytosis did not kill bacteria which makes it probable together with data[29] on the rate at which neutrophils leave the blood stream that the function of the neutrophils is to clear the blood stream of organisms and to localize infection and that the killing of organisms depends on immune mechanisms and not on phagocytosis

Studies of mitosis by Gunz[30] and our own group with or without addition of colchicine showed that all that is necessary to lead to mitosis of the progranulocytes of leukemic blood is the dilution of the blood serum with a simple balanced salt solution of the types commonly used in tissue culture[6]

Studies of the action of ionizing radiation[31,32,33] of various modalities (X ray neutrons I[?]) showed that in doses under 400 r or its equivalent for the granulocytic series and under 50 r for the lymphocytic series the effects could be explained entirely by inhibition of mitotic rate and that there was no evidence of killing of cells By interchange of media[33] it was shown that the effects were direct and not due to any humoral factor that was stable for more than a few minutes These effects on the granulocytic series could be quantitatively duplicated with colchicine in the cultures and they could not be explained by the no threshold[34] action of ionizing radiation in producing chromosome and gene changes but were due to an inhibition of mitosis occurring during interphase which prevented or delayed the onset of the next mitosis Based on these studies a treatment called titrated regularly spaced total body irradiation[35] was developed for chronic leukemias which has approximately doubled the survival time of such patients and when combined with cortisone* therapy approximately doubles the survival time of acute leukemias[36]

* W d b d t M k nd Compa y I p d g tl t son d hyd or rt sc

TISSUE CULTURE IN THE STUDY OF LEUKOCYTIC FUNCTIONS*

By Edwin E Osgood

Division of Experimental Medicine University of Oregon Medical School Portland Ore

Early work on the culture of leukocytes has been reviewed and illustrated by Fieschi and Astaldi [1] who cite over 340 references and by Bloom [2] Our own early work has also been reviewed [3 4 5] so that only a few of the more important recent developments will be considered here

The double cover slip and hanging drop tissue culture techniques [6 7 8] with or without a plasma clot have given valuable information on the motility living morphology and mitotic activity of leukocytes and are well adapted to phase microscopy and time lapse moving picture studies The classic work of Maximow and Bloom was done by these methods and has been reviewed by Bloom [9] It was the time lapse moving picture studies on such preparations by Rich Wintrobe and Lewis[10] which made possible the first complete description of the method of locomotion of the different series of leukocytes The double cover slip technique when combined with a perfusion device such as that described by Pomerat [11] permits the direct observation or time lapse photography under phase microscopy of the action of many variables on living leukocytes in culture The action of fixatives and various poisons as described by Hsu and Pomerat[12] could well be applied to this technique By exposing such cultures to hypotonic Gey s solution and then to a fixative Hsu[13] has shown that human leukocytes as well as many other cells show marked degrees of aneuploidy[14] and polyploidy and has published some of the most beautiful pictures extant of human chromosomes [15]

The roller tube method of tissue culture[16] which has been so successful in the culture of other types of cells has with a single exception proved to date unsatisfactory for the growth of hemic cells This single exception however is very important for de Bruyn [17] while working in Gey s laboratory was the first to grow leukocytes of any series other than the monocytic over long periods of time The strain of lymphoblasts she started in culture from a transplantable mouse lymphosarcoma is still growing well after more than five years [18] Bichel[19] has also succeeded in growing mouse hemic cells in hanging drop cultures in a sort of symbiosis with actively growing fibroblasts The cells he cultured were leukemic lymphocytes and plasmocytes of the mouse derived from transplantable leukemias and these cells have been cultured through many passages and over long periods of time

A continuous flow large scale culture device [20] has given the longest cell survival (58 days) of any method developed prior to the gradient culture for the granulocytic series derived from human marrow The Carrel and Lindbergh[21] whole organ culture technique in which a continuous pump simulating heart action was connected to the nutrient arteries of a segment of bone with marrow

* Th w k w ppo t d i p t by g nt f m th M d l R se h F d t f O g th L t d St t Atomic Energy Comm s a d by gr t N C 820 C 1955 d CS 9268 f m th N t l C f t t t N t l In t t t f H lth U t d St t P bl H lth S

ture in an incubator will have the same life span in the same vial culture and (2) that changes in numbers of differentiating cells are straight line arithmetic functions it is possible to calculate by a graphic method the length of life of differentiating neutrophils from the data obtained in the studies of cortisone and hydrocortisone [35] Such calculations[43] lead to a life span of only 67 hours for the neutrophils of chronic granulocytic leukemia as compared to 107 hours for the neutrophils from nonleukemic marrow The data on the method of division and length of life of the cells made possible a computation that only 1/40 to 1/400 of the cells outside the blood forming organs are present in the blood stream at any one moment of time in standard man [29]

These examples chiefly drawn from our own work illustrate the great possibilities of this method for short term (8 to 10 days) quantitative controlled studies but indicate also that the rate of cell division in these cultures does not keep pace with the rate of cell differentiation and cell death

Many unpublished studies on variations of oxygen tension composition of medium and number of cells per unit volume of medium have failed to solve the problem of obtaining long term continuous multiplication of cells with increase in number but had shown that the number of cells per unit volume controlling pH between 7 2 and 7 6 and the oxygen tension were all critical factors With any medium tried at depths under 2 cm and cell counts under 2 000 all cultures showed drops to about 20 per cent of their initial count by 8 to 12 days and few or no living cells by 20 to 30 days

The development of the gradient culture[44] explained the previous failures to obtain multiplication of hemic cells and also the few successes Why it should have taken me 18 years to think of anything so simple I shall never understand It consists of placing a clean sterile 1 × 3 inch glass slide at an approximately 45° angle in a one pint French square bottle containing 150 ml of suspension type culture in 40 per cent serum and 60 per cent TC 199[45]* or probably any other of the frequently used fluid tissue-culture media with a suspension of cells from leukemic blood or hematopoietic organs of about 1000 per cu mm and incubating at 37° C The supernatant gas mixture is air with enough CO_2 (usually about 5 per cent) to hold the pH at the desired level As the cells settle on the slide the numbers steadily increase from above downward to the depth of 5 cm and the distance from the source of oxygen steadily increases so that there is a continuous gradient in oxygen tension oxidation reduction potential and number of cells per unit volume of medium and at some point conditions are certain to be right Furthermore there is an opportunity for the more motile mature cells to migrate over the edge of the slide Even with the very simple medium used in the cortisone and hydrocortisone studies which is inferior to TC 199 growth far superior to anything we had previously observed even in continuous flow cultures has been noted in every type of leukemic blood or normal or neoplastic marrow so far investigated Slides could be removed with sterile forceps at any desired interval rinsed with culture media as they were withdrawn and then rinsed with balanced salt solution to remove loosely adherent cells and excess protein and next be examined while

Quantitative studies of the effects of nitrogen mustard[33] showed that these effects were due to killing of cells, not merely inhibition of cell division and that the minimum effective concentration and the maximally effective concentration were very close together. Studies of the action of urethane[37] demonstrated karyorrhexis of the nucleus as the most characteristic effect with a tendency to formation of double nucleated cells. Quantitative studies of the effects of cortisone and hydrocortisone[38] in such cultures showed that these agents, even in concentrations 10 times those attainable clinically, did not affect the rate of cell division, cell death, or differentiation of any type of leukocyte tested in these cultures, but the prolymphocytes and lymphoblasts of acute leukemia on which these agents are clinically most effective were not investigated because they promptly disintegrated in such cultures at shallow depths of less than 2 cm.

In unpublished studies, Miss Migaki, a graduate student in our laboratory, compared the disintegrated cell count with supravital studies of motility and phagocytic ability, and with Schrek's method[39] of nuclear staining by safranin. These studies showed that the disintegrated cell count is the most accurate method thus far available of recognizing cells which are dead but have not as yet undergone complete autolysis, but that still better methods are needed. Disintegrated cell counts parallel the Schrek stained nuclei count, but at a higher level.

Studies of the uptake of radioactive phosphorus in cultures[40] containing as much as 10 gm. or 20 billion cells from chronic granulocytic leukemia in one liter of medium showed that the radioactive phosphorus was incorporated into the DNA and provided further evidence that new formation of cells does occur in this type of culture. Cultures of the blood of healthy medical students[41] and their marrows under identical conditions showed that the length of life of the segmented neutrophil is of the order of 48 hours and that of the neutrophils and band cells present in the blood is about 60 hours, suggesting that the time of entrance of the cells of the granulocytic series into the blood stream is 60 hours less than the total time to cell death.

All of our data on quantitative differentiation in these cultures indicated that division occurred only in cells containing nucleoli (in other words, in the blast, pro-, and early myelocyte stages) and that changes in numbers of cells more mature than these (in other words, in the differentiating cells), followed straight line arithmetic curves. This could also be theoretically deduced in that if a cell reaches a stage at which it could no longer divide, it should be obvious that if both cells resulting from the division remain immature to divide again, there will be no cell to differentiate, and if both differentiate, no cell will remain to divide.[4] Therefore, from any division one cell must remain immature and, for practical purposes, be the same cell, and the other must differentiate. It is true that at rare intervals divisions may occur in which both cells remain immature to divide again, but in this case changes in cell number will always be logarithmic.

If we make then but two assumptions: (1) that the neutrophil myelocytes resulting from a division immediately prior to aspiration of the blood or marrow and those resulting from a cell division immediately after placing the cul

ture in an incubator will have the same life span in the same vial culture and (2) that changes in numbers of differentiating cells are straight line arithmetic functions it is possible to calculate by a graphic method the length of life of differentiating neutrophils from the data obtained in the studies of cortisone and hydrocortisone[33] Such calculations[43] lead to a life span of only 6? hours for the neutrophils of chronic granulocytic leukemia as compared to 102 hours for the neutrophils from nonleukemic marrow The data on the method of division and length of life of the cells made possible a computation that only 1/40 to 1/400 of the cells outside the blood forming organs are present *in the blood stream at any one moment of time in standard man* [9]

These examples chiefly drawn from our own work illustrate the great possibilities of this method for short term (8 to 10 days) quantitative controlled studies but indicate also that the rate of cell division in these cultures does not keep pace with the rate of cell differentiation and cell death

Many unpublished studies on variations of oxygen tension composition of medium and number of cells per unit volume of medium have failed to solve the problem of obtaining long term continuous multiplication of cells with increase in number but had shown that the number of cells per unit volume controlling pH between 7 2 and 7 6 and the oxygen tension were all critical factors With any medium tried at depths under 2 cm and cell counts under ?000 all cultures showed drops to about 20 per cent of their initial count by 8 to 12 days and few or no living cells by 20 to 30 days

The development of the gradient culture[44] explained the previous failures to obtain multiplication of hemic cells and al o the few successes Why it should have taken me 18 years to think of anything so simple I shall never understand It consists of placing a clean terile 1 × 3 inch glass slide at an approximately 45 angle in a one pint French square bottle containing 170 ml of suspension type culture in 40 per cent serum and 60 per cent TC 199[45]* or probably any other of the frequently used fluid tissue culture media with a suspension of cells from leukemic blood or hematopoietic organs of about 1000 per cu mm and incubating at 37 C The supernatant gas mixture is air with enough CO_2 (usually about 5 per cent) to hold the pH at the desired level As the cells settle on the slide the numbers steadily increase from above downward to the depth of 5 cm and the distance from the source of oxygen steadily increases so that there is a continuous gradient in oxygen tension oxidation reduction potential and number of cells per unit volume of medium and at some point conditions are certain to be right Furthermore there is an opportunity for the more motile mature cells to migrate over the edge of the slide Even with the very simple medium used in the cortisone and hydrocortisone studies which is inferior to TC 199 growth far superior to anything we had previously observed even in continuous flow cultures has been noted in every type of leukemic blood or normal or neoplastic marrow so far investigated Slides could be removed with sterile forceps at any desired interval rinsed with culture media as they were withdrawn and then rinsed with balanced salt solution to remove loosely adherent cells and excess protein and next be examined while

* Obta ble f om D fco L bo to D t t M h

living or fixed by any method and stained with any stain After cell counts on the mixed culture, a new slide could be introduced

It was soon found that the position of cells on the slide could be varied by varying the number innoculated or the depth of the layer, so that the conditions for optimal growth of each cell type could best be described by a 'gradient factor,' which is defined as the cell count in thousands per cu mm times the depth below the surface in centimeters at which growth is best when the gas mixture is room air The gradient factor for the various types of leukocytes varied from about 1 5 for the plasmocytic series to 12 0 for the erythrocytic series with the granulocytic monocytic and lymphocytic series at about 3 5 4 5 and 6 0 in between The gradient factors for the monocytic series completely overlapped those for the granulocytic series so that these cells were not separable although individual colonies were of one cell type

The pattern of growth of each type of cell was characteristic and resembled that of the corresponding cells in the hematopoietic organs (see plates 1-3 in reference 44) Since these patterns are developed within 24 hours it seems probable this method will prove valuable in identifying the cell type in acute leukemias where such identification is notoriously difficult Increases of two to five times the number of cells of a particular type have been obtained in cultures of leukemic human blood or plasmocytic myeloma marrow for the first time by this method and it seems possible that pure strains of cells may be isolated Merely tipping the bottle at a 45° angle without a gradient slide, while giving results far superior to our previous methods of culture at depths below 2 cm gives results definitely inferior to those with the gradient slide present This finding together with our previous studies suggested that removal of the mature cell was an important factor in the success of the method

Applying the principles deduced from this gradient culture, John Brooke cytologist in the Division of Experimental Medicine has obtained multiplication of cells over periods in excess of 60 days and by a factor of over five times the number of cells initially introduced in rectangular flat sided 4 ounce pharmaceutical bottles capped with vaccine caps and laid with the flat side down Reasoning that the gradient depended both on depth and number of cells and that by a high cell count we could obtain a high gradient factor with a relatively thin layer and noting on the gradient slides that the immature cells adhered to glass while the more mature cells did not the following modification of the vaccine vial capped suspension culture was developed Heavy inocula of 15 to 30 thousand cells per cu mm were made into a volume of 10 to 12 ml of fluid medium in bottles This volume gives a layer only 3 to 4 mm deep These cells were allowed to rest on the flat side in the incubator for 1 to 16 hours so that the immature cells might affix themselves to the glass wall and then the more mature cells were removed after very gentle rocking of the bottle to place them in suspension The number remaining in the bottle were determined by the difference in leukocyte count Growth could be observed by direct microscopy through the wall of the bottle and by pH change and subcultures were made from the cells removed at intervals of one to seven days depending on the degree of multiplication From the blood of one patient with terminal chronic granulocytic leukemia we attained at 30 days 10 such cul

tures with a total cell count of at least 3 5 times the number initially inoculated These cells are not morphologically normal but are living as demonstrated by phase microscopy at 72 days and are peroxidase positive They resemble progranulocytes but there are also many giant cells and fibroblast like cells probably derived from the monocytic series

Many variations on the gradient technique are obviously possible Perforated cellophane sheets may be supported on a glass rod frame at a similar angle or several progressively narrower layers may be wrapped around the gradient slide to give many gradients A narrow strip of Leighton sponge[46] *held in place by a plasma clot along the center of the long axis of the gradient* slide seems best adapted for separation and identification of metastatic neoplastic cells from marrow The development of a device for continuous flow with continuous control of all variables seems most promising for long term production of new cells of any desired type A preliminary model designed largely by Doctor Demetrios Rigas physical chemist and biochemist in the Division of Experimental Medicine at the University of Oregon Medical School when used with a gas mixture of room air diluted 50 per cent with nitrogen has given marked increases in the plasmocytic series when compared with the initial absolute number present in the two marrows of healthy members of our staff that have been investigated One of these cultures has now begun to show increases in cell count after the initial drop Living cells are still present at 50 days More time must of course elapse before we can say that really long term cultivation of human hemic cells is possible but the prospects seem promising

These studies indicate clearly that the progranulocytes and promonocytes and prolymphocytes of leukemic blood are capable of division to produce cells capable of both division and differentiation They strongly suggest that not only hemic cells but other cell types also may grow best at certain definite oxygen tensions and oxidation reduction potentials and that it would be desirable to repeat metabolic studies under such ideal oxygen tensions They also indicate that the chemically mature cell of these series produces a relatively unstable inhibitor of cell division and there is much ancillary evidence that this finding may be true in other types of cells which differentiate beyond the stage capable of division This postulated humoral mechanism of homeostasis for hemic and other cells which differentiate has its analogues in the inducers and organizers of embryologic development which also appear to be short range relatively unstable substances produced by the living cell

A hypothesis as to the fundamental mechanism of leukemia and cancer has been deduced from the studies described and will be presented in more detail elsewhere If all that were necessary for the production of unlimited growth in other words cancer were absence of an inhibitor produced by the chemically mature cell then *any* change in the cell capable of division whether it be chromosomal genic or plasmagenic which would lead to a shorter life span of the differentiating cell would constitute the necessary difference from the normal to be called cancer If this were true all of the current theories (heredity viral chemical or physical carcinogen) of the mechanism of cancer production

may be correct. This hypothesis would account for the different enzyme constitution of different cancers of apparently identical type and thus unify all our present knowledge in regard to cancer. It would also change the direction of cancer research, in that we should study the normal mature cell of the series in attempts to isolate a relatively unstable inhibitor of mitosis which should prove to be theoretically the most efficient agent for the treatment of leukemia or cancer of the same cell series. This hypothesis would also explain the tendency of malignant tumors and leukemias to become more acute or anaplastic with time, since the rapid multiplication would statistically increase the chances of a second or third mutation or chromosome change. Such a development would still further shorten the life span and, at some point, would cause cell death before development of an hypothetical inhibitor of the logarithmic type of division which occurs when cells divide to form two cells which may divide again.

The type of division described for hemic cells would explain the picture that has often been interpreted as maturation arrest as due, instead, to early cell death. It would explain what has often been thought to be dedifferentiation on the same basis and would explain some cases of metaplasia as due to a differential rate of multiplication. The hiatus leukemicus would represent, in many cases, merely temporary decrease in division rate followed by more rapid division.

Past studies of the physiology of the human leukocytes by tissue culture methods have given much valuable quantitative information, but methods previously available have had serious limitations. The development of the gradient culture and its modifications offers promise of extending the usefulness of tissue culture for the study of the physiology of leukocytes and other human cells. Human leukocytes from leukemic blood in suspension type cultures have the great advantage for enzyme and metabolic studies of not being contaminated with other tissue cells such as artery, veins, connective tissue. Moreover, the cells have not been cut. A unifying concept of leukemia and cancer is suggested. It seems probable that the development of new tissue culture tools will make progress in the study of the physiology of leukocytes much more rapid in the future than it has been in the past.

A survey of the sections on leukemia, leukocytes, marrow, lymph nodes and spleen in the wonderfully valuable contribution called *A Bibliography on the Research in Tissue Culture*[47] prepared by Margaret K. Murray and Gertrude Kopech will show what a very small fraction of the total work on tissue culture in the study of leukocytes it has been possible to review, and what a small fraction of this total work is represented by our investigations, from which most of the examples of applications have been drawn simply because illustrative material was more readily available.

References

1. FIESCHI, A. & C. ASTALDI. 1946. La cultura in vitro del midollo osseo. Problemi de fisiopatologia ematologica studiati con la tecnica della cultura dei tessuti. Haematologica 8: 1.
2. BLOOM, W. 1938. Tissue cultures of blood and blood forming organs. In Handbook of Hematology. 1469-1585. H. Downey, Ed. Hoeber. New York, N. Y.

3 Osgood F F 1939 The culture of human marrow as an aid in the evaluation of therapeutic agents J Lab Clin Med 24 954
4 Osgood E E 1939 Marrow cultures In A Symposium on the Blood 219-241 University of Wisconsin Press Madison Wis
5 Osgood E E 1940 Culture of human marrow summary of stud es to date West ern J Surg 48 540
6 Parker R C 1950 Methods of tissue culture 2nd ed Harper & Brothers New York N Y
7 Pomerat C M *et al* 1951 Tissue culture methods In Methods in Medical Re search 4 198-291 M B Visscher Ed The Year Book Publishers Chicago Ill
8 Cameron G 19 0 Tis ue Culture Technique Academic Press New York N Y
9 Bloom W 1937 Cellular differentiation and tissue culture Physiol Revs 17 589
10 Rich A R M M Wintrobe & M R Lewis 1939 The differentiation of myeloblasts from lymphoblasts by their manner of locomotion A motion picture study of the cells of normal bone marrow and l mph nodes and of leukemic blood Bull Johns Hopkins Hosp 65 291
11 Pomerat C M 1951 Methods in Med cal Research 4 215-277 M B Visscher Ed The Year Book Publishers Chicago Ill
12 Hsu T C & C M Pomerat 1953 Mammalian chromosomes in vitro II A method for spreading the chromosomes of cells in tissue culture J Heredity 44 23
13 Hsu T C 1952 Mammalian chromosomes in vitro I The karyotype of man J Heredity 43 167
14 Hsu T C & C M Pomerat 1953 Mammal an chromosomes in vitro III On somatic aneuploidy J Morphol 93 301
15 Hsu T C 1954 Mammal an chromosomes in vitro IV Some human neoplasms J Nat Cancer Inst 14 905
16 Gey G O 1933 An improved techn c for massive tissue culture Am J Cancer 17 752
17 De Bruyn W M & G O Gey 1952 Further studies in vitro of transplantable mouse lymphosarcoma MB (T 86157) Acta Union Intern contre le Cancer 7 712
18 Gey G O Personal communication
19 Bichel, J 1952 Cult vation of leukemic cells in tissue culture Acta Path Microbiol Scand 31 410
0 Osgood E E 1938 Culture of human marrow an improved apparatus for large scale culture Am J Med Sci 195 141
21 Carrel A & C A Lindbergh 1938 The culture of organs Hoeber New York N Y
22 Tullis J L Personal communication
23 Osgood E E & I E Brownlee 1937 Culture of human marrow details of a simple method J Am Med Assoc 108 1793
24 O good E F 1938 Culture of human marrow a comparati e study of the effects of sulfanilamide and antipneumococcus serum on the course of experimental pneumococci infections Arch Internal Med 62 181
25 Bullowa J G M E E Osgood S C Bukantz & I E Brownlee 1940 The effect of sulfapyridine alone and with serum on pneumococcic pneumonia and on pneu mococcus-infected marrow cultures Am J Med Sci 199 364
26 Osgood E E 1940 Culture of human marrow stud es of the relative effecti eness of neoarsphenamine mapharsen sulfan lam de sulfapyr dine sulfathiazole and sulfa methylthiazole on infections with streptococcus viridans (alpha hemolytic treptococ cus) Am J Med Sci 200 596
27 Osgood E E 1942 Comparative effectiveness of neoarsphenamine sulfathiazole sulfadiazine sulfapyridine sulfanilamide and other sulfonamide drugs against Ne s seria Gonorrhoeae in marrow cultures Am J Syphilis Gonorrhea Veneral Diseases 26 133
28 O good E E 1938 Culture of human marrow studies of mode of act on of sul fanilamide J Am Med Assoc 110 340
29 Osgood E E Number and distribution of human hemic cells Blood 9 1141
30 Gunz F W 1948 Culture of hum n leukaemic blood cells *in vitro* technique and the growth curve Brit J Cancer 2 29
31 Osgood E E P C Aebersold L A Erf & E A Packham 1942 Studies of the effects of mill on v lt roentgen rays 200 kilovolt r entgen rays rad oacti e phosphorus and neutron rays by the marrow culture techn que Am J Med Sci 204 372
32 Osgood E E 1942 Is the act on f roentgen rays d rect or indirect An investigation of this question by the method of human marrow culture Am J Roentgenol Radium Therapy 48 214

may be correct This hypothesis would account for the different enzyme constitution of different cancers of apparently identical type and thus unify all our present knowledge in regard to cancer It would also change the direction of cancer research in that we should study the normal mature cell of the series in attempts to isolate a relatively unstable inhibitor of mitosis which should prove to be theoretically the most efficient agent for the treatment of leukemia or cancer of the same cell series This hypothesis would also explain the tendency of malignant tumors and leukemias to become more acute or anaplastic with time since the rapid multiplication would statistically increase the chances of a second or third mutation or chromosome change Such a development would still further shorten the life span and at some point, would cause cell death before development of an hypothetical inhibitor of the logarithmic type of division which occurs when cells divide to form two cells which may divide again

The type of division described for hemic cells would explain the picture that has often been interpreted as maturation arrest as due instead to early cell death It would explain what has often been thought to be dedifferentiation on the same basis and would explain some cases of metaplasia as due to a differential rate of multiplication The hiatus leukemicus would represent in many cases, merely temporary decrease in division rate followed by more rapid division

Past studies of the physiology of the human leukocytes by tissue culture methods have given much valuable quantitative information but methods previously available have had serious limitations The development of the gradient culture and its modifications offers promise of extending the usefulness of tissue culture for the study of the physiology of leukocytes and other human cells Human leukocytes from leukemic blood in suspension type cultures have the great advantage for enzyme and metabolic studies of not being contaminated with other tissue cells such as artery veins connective tissue Moreover the cells have not been cut A unifying concept of leukemia and cancer is suggested It seems probable that the development of new tissue culture tools will make progress in the study of the physiology of leukocytes much more rapid in the future than it has been in the past

A survey of the sections on leukemia leukocytes marrow lymph nodes and spleen in the wonderfully valuable contribution called *A Bibliography on the Research in Tissue Culture*[47] prepared by Margaret R Murray and Gertrude Kopech will show what a very small fraction of the total work on tissue culture in the study of leukocytes it has been possible to review and what a small fraction of this total work is represented by our investigations from which most of the examples of applications have been drawn simply because illustrative material was more readily available

References

1 FIESCHI A & G ASTALDI 1946 La cultura in vitro del midollo osseo Problemi de fisiopatologia ematologica studiati con la tecnica della cultura dei tessuti Haematologica 8 1

2 BLOOM W 1938 Tissue cultures of blood and blood forming organs. In Handbook of Hematology 1469–1585 II Downey Ed Hoeber New York N Y

THE PROTECTIVE EFFECT OF GRANULOCYTES IN RADIATION INJURY*

By E. P. Cronkite and George Brecher

United States Naval Medical Research Institute and National Institutes of Health, Bethesda, Md.

Historical Background

The destructive effects of whole body irradiation on blood forming tissues were recognized by Heinecke in 1903 and described in a series of papers[1,2] to which little of a qualitative nature can be added even today. Heinecke's observation of the extreme sensitivity of lymphoid tissues suggested the use of local irradiation for lymphoid tumors and the attention of many investigators became focused on the blood changes following radiotherapy, best demonstrated by Minot and Spurling.[4]

The development and use of atomic bombs renewed interest in the effects of whole body radiation. Military and peaceful application of atomic energy gave particular impetus to studies on protection against radiation injury. Furthermore, as a result of the extensive applied studies, it has been found that radiation, when properly controlled, can be an excellent tool to aid in the study of basic physiologic problems concerned with hematopoiesis, formed elements of the blood, and hemostasis. In this article, however, the authors will limit themselves to a brief summary of the over-all effects of whole body irradiation and a discussion of the specific relationship of leukopoiesis, infection, and mortality. Many detailed reviews[6-16] on all aspects of the effects of radiation are available.

Whole Body Radiation Syndrome

Death following irradiation can be due to several lethal syndromes which are schematically portrayed in FIGURE 1. The first wave of deaths occurs under the beam or within 24 to 48 hours and is termed *neurological* because of the characteristic symptomatology.[17,20] The second wave of deaths, which occurs at lower doses when death is not produced by the neurologic syndrome, has been termed the *gastrointestinal syndrome* because of the GI symptoms and severe histopathologic lesions of GI tract.[12,18,21,6] When death is produced by this syndrome, it occurs between the second and eighth days. Death from the gastrointestinal syndrome is rare below [illegible]50 r. Above 1200 r, the syndrome is uniformly fatal with a mean survival time of about four days in mice, rats, and dogs. In hamsters, goats, and guinea pigs, deaths rarely occur as early as four days. The longer survival time after large doses has been ascribed to a large colon[1,23] that apparently retains its capacity as a water absorber, thus preventing the severe loss of water and electrolytes such as occurs in the species with the shorter survival time after the same doses. The third syndrome has been termed the *hemopoietic syndrome* because it is characterized by bone marrow aplasia. The resulting pancytopenia leads to infection, hemor-

* [illegible]

33 Osgood E E & I T Chu 1950 The effect of nitrogen mustard upon the granulocytic cells as observed by the marrow culture technic Cancer Research 10 98
34 Muller H J 1950 Radiation damage to genetic material Am Scientist 38 33
35 Osgood E E & A J Seaman 1952 Treatment of the chronic leukemias results of therapy of 163 patients by titrated regularly spaced total body radioactive phosphorus or roentgen irradiation J Am Med Assoc 150 1372
36 Osgood E E A J Seaman & H Tivey 1953 Etudes radiobiologiques sur les cellules du sang chez l'homme Rev d hemat 8 403
37 Osgood E E & I T Chu 1948 The effect of urethane on the nuclear morphology of cells of the granulocyte series Blood 3 911
38 Krippaehne M L & E E Osgood Studies of the influence of cortisone and hydrocortisone on human leukocytes in culture and eosinophilic leukemia Acta Haemat In press
39 Schrek R 1944 Studies in vitro on the physiology of normal and of cancerous cells II The survival and the glycolysis of cells under aerobic and under anaerobic conditions Arch Path 37 319
40 Osgood E E J G Li H Tivey M L Duerst & A J Seaman 1951 Growth of human leukemic leucocytes *in vitro* and *in vivo* as measured by uptake of P^{32} in desoxyribose nucleic acid Science 114 95
41 Osgood E E 1937 Culture of human marrow length of life of the neutrophils eosinophils and basophils of normal blood as determined by comparative cultures of blood and sternal marrow from healthy persons J Am Med Assoc 109 933
42 Osgood E E 1953 Drug induced hypoplastic anemia and related syndromes Ann Internal Med 39 1173
43 Osgood E E & M L Krippaehne Comparison of the life span of leukemic and nonleukemic neutrophils Acta Haemat In press
44 Osgood E E & M L Krippaehne The gradient tissue culture method Exptl Cell Research In press
45 Healy G M & R C Parker 1953 Preparation of synthetic mixture 199 Revised procedure March 1953 Mimeographed copy
46 Leighton J 1951 A sponge matrix method for tissue culture Formation of organized aggregates of cells in vitro J Natl Cancer Inst 12 545
47 Murray Margaret R & Kopech Gertrude 1954 A Bibliography of the Research in Tissue Culture (2 vols) Academic Press New York N Y

TABLE 1
LETHAL DOSES FOR VARIOUS MAMMALS

Spe	D se l r t p od m t l t 30 d ys		R d t h t t s
	50%	0 100%	
Dogs	312	180–555	2 0 mev HVL 4 3 mm Pb 15 r/min in air
Swine	400	250–525	2 0 mev HVL 4 3 mm Pb 15 r/min in air
Guinea pigs	250	150–500	200 KVP 1 mm Al 0 25 mm Cu HVL 0 8 mm Cu 44 r/min in air
Monkeys	528 ± 12	425–630	230 KVP HVL 1 25 mm Cu 3 r/min in air
Rats Sprague-Dawley 100 gm	4/8	350–665	200 KVP 1 mm Al 0 25 mm Cu HVL 0 8 mm Cu 44 r/min in air
Rats 150 gm	725	400–800	Cobalt 10 r/min in air
LAf Mice	754 ± 8	500–950	2 0 mev HVL 4 3 mm Pb 15 r/min in air
Hamster	710	540–975	200 KVP 20 ma 0 51 mm Al 25 mm Cu 27 r/min in air

D t bt d f m ref 29 3

TABLE 2
MEAN LEUKOCYTE COUNTS IN VARIOUS SPECIES IN THOUSANDS

Spe	T t l l kocyt × 10³	G locyt × 10	Lymph d m cyt × 10
Dog	12 7	10 3	2 4
Swine	17 0	4 6	12 4
LAf Mice	8 4	1 2	7 2
C 15 Mice‡	9 6	2 1	7 4
Man	7 8	5 3	2 5
Hamster†	10 5	2 2	7 0
Monkey	13 2	4 0	9 2

P so l d t
† W W Sm th (P so l mm t)
‡ T g J h d J q (37)
P tt so (30)

and species differences in leukocyte response were evident but not fully appreciated

In making species comparisons it is essential to compare absolute numbers of specific cell types rather than total white blood count because of the great differences in the proportion of lymphocytes and granulocytes in the peripheral blood of various species as illustrated in TABLE 2

Lymphocytes are the most sensitive leukocytes in their response to irradiation and they behave similarly in all species The lymphocyte decrease begins immediately after exposure and minimum values are obtained within 36 to 48 hours most of the decrease occurring within 24 hours of exposure The rate of decrease as a percentage of lymphocytes present at time of exposure approximates a linear regression from the time of exposure[10] Within any species the recovery time for lymphocytes is usually much longer than for granulocytes however in LAf_1 mice recovery is similar

In contrast to lymphocytes the granulocytes respond with an initial rise[5, 9]

LETHAL SYNDROMES AFTER VARIOUS DOSES OF RADIATION

NM
>30 000r

GI
1000 30 000r

HEMOPOIETIC
200 1000r Hemorrhage
Anemia
Infection

LATE EFFECTS
Leukemia
Cancer
Longevity<

No DYING

Hours
3 6 9 12 15 DAYS
20 80 160 WEEKS

TIME AFTER IRRADIATION

FIGURE 1 Sch m t p t y l f l th l y d me t m f fd th d to d ff e ts y d m s d d s ge th t pr d c th y d m m po d to 2000 KVP X y with dos te betw 15 r d 2000 r pe m n

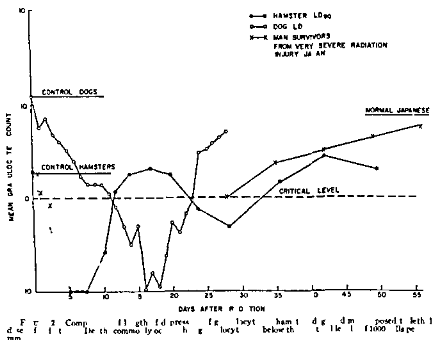
rhage and anemia all three of which may kill [6] However infection occurs earlier than hemorrhage and anemia and accordingly plays the dominant role

In most species the GI syndrome occurs only at supralethal doses and deaths in the lethal dose range* are predominantly hemopoietic However in rats a fairly severe GI syndrome may occur between the LD_{50} and LD_{100} (see TABLE 1) with some deaths on the third and fourth days Animals that recover from this GI syndrome subsequently develop pancytopenia Thus two peaks *of deaths may result from the same dose the first around the fourth day* being correlated with sensitivity of the GI tract [8] the second with the bone marrow damage Observation in the Japanese indicates that man is also subject to the double hazard of the GI syndrome and later pancytopenia [27]

Changes in Granulocyte Count as a Function of Species Dose and Time after Irradiation

Under normal conditions the various formed elements are at a relatively constant level denoting a dynamic equilibrium between production and use Radiation obviously disturbs this equilibrium Apparently the first dose time and species comparative study was that of Taylor *et al* [5] During the development of the atomic bomb extensive comparative studies were made [8 10 33] These studies were extended during atomic bomb experience [34 36]

The t rm l thal d se d t d t d se lt g d th f l t 99% f th po d m l
I nt t the t m p l thal d t d t un f rmly l th l d se



F r 2 Comp f l gth f d press f g l cyt ham t d g d m posed t leth l d se f l t De th commo ly oc h g locyt below th t l le l f 1000 lls pe mm

between speed of recovery of granulocytopoiesis and probability of recovery as will be discussed later

In FIGURE 3 are shown the changes in granulocytes in the dog after different doses of radiation After 150 r a sublethal dose the depression is slight and recovery is just about complete by 25 days After 300 r, an approximate LD_{50} the depression is moderate the count undulates and recovery is fairly complete by 30 days After 400 r an LD_{88} the fall continues for days reaching minimum values between 16 to 18 days after exposure In those animals that survive the recovery is slow and not complete for more than 30 days After 600 r an LD_{100} the fall is more rapid * and minimum granulocyte levels of less than 500 per cu mm are reached by 5 to 7 days and remain below this level or completely disappear before death 7 to 21 days after exposure

The Susceptibility to Infection Its Relation to Mortality and the Granulocyte Count

Warren and Whipple[45] and Lawrence and Tennant[46] working with high doses of radiation that produced death in a few days concluded that infection is not an important factor in roentgen ray deaths This conclusion was confirmed by Osborne *et al*[47] in mice with large doses (2000–3000 r) Gonshery *et al*[48]

* Th d p lm t m m l t th d se f th d g Aft 3000 m l m y d th d f th l bef m m m dep h d Afte 10 000 m d gs d bef le k pe h tum t l l p

TABLE 3

TIME OF APPEARANCE OF MAXIMUM DEPRESSION AND RECOVERY TO NORMAL IN GRANULOCYTES FOR VARIOUS SPECIES AND REPRESENTATIVE DOSES OF X RAY

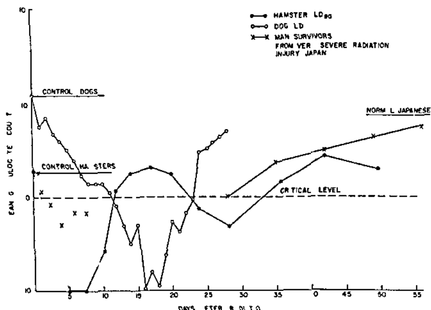
Species	Dose in r	Per cent mortality	Time to maximum depression in days	Time to recovery to normal in days
Rat	500	50	5	20
	500	—	4	—
	500	—	3–4	25
	550	50	3–5	16–20
	650	68	2	—
	800	90	4	30–40
	800–1500	100	3	—
Mouse	600	—	4–6	26–30
LAf1	650	—	4–6	26–30
	800	80	5	20–30
Dog	150	0	14–16	30
	300	50	13 & 19	>30
	400	88	16	>30
	600	100	5–10	—
Monkey	528	50	6–10	Most by 26 days some 41 days
Cat	200	—	3–4	40–50
Hamster	825	90	5–7	15
Swine	400	12	6 & 21	60–90
	600	8[illegible]	5	60–120
Man	[illegible]	50?	5–10 ? & 24	—

Data assembled from references 9 10 11 27 30 33 3[illegible] 3[illegible] 37 38 39 40 41 42 43 44

The granular leukocytosis* consisted of two peaks one at 8 to 12 hours and a later one at 24 hours Thereafter there was a decrease The magnitude of the depression and within limits of the duration was a function of the dose of radiation This primary depression may be followed by an abortive rise[1] and a secondary depression clearly described in the rabbit by Jacobson *et al.*[9] In TABLE 3 are tabulated the time to maximum depression and time for recovery to normal for the granulocytes in various species after various doses of radiation There is relatively little difference in the rate of development of the primary granulocytopenia for comparable doses of radiation However at the same per cent mortality the granulocytopenia develops more slowly in the dog There are marked species differences in the rate of recovery The hamster recovers most rapidly and man and swine most slowly In FIGURE 2 the relative rates of depression and recovery in the granulocytes are plotted for the hamster and the dog at an approximate LD_{90} for both species The human data are from a group that approximates an LD_{60} [40] The very rapid recovery of granulocytes in the hamster† is evident The intermediate recovery rate in the dog and the very sluggish recovery in man is apparent Since the mortality rates for the three species are comparable in the present instance it is apparent that survival is compatible with different duration of granulocytopenia in different species A general correlation exists however

* S[illegible] lethal dose [illegible] [illegible] omitted from this table

† Hamster data kindly supplied by Doctor W. W. Smith, United States National Institutes of Health.



FIGU 2 Comp ≈ f l gth f d pre f g locyt h m t d g d m p sed t l th l d se f l t D th mm ly oc wh g locyt bel w th t l l f 1000 lls pe mm

between speed of recovery of granulocytopoiesis and probability of recovery as will be discussed later

In FIGURE 3 are shown the changes in granulocytes in the dog after different doses of radiation After 150 r a sublethal dose the depression is slight and recovery is just about complete by 25 days After 300 r an approximate LD_{50} the depression is moderate the count undulates and recovery is fairly complete by 30 days After 400 r an LD_{88} the fall continues for days reaching minimum values between 16 to 18 days after exposure In those animals that survive the recovery is slow and not complete for more than 30 days After 600 r an LD_{100} the fall is more rapid * and minimum granulocyte levels of less than 500 per cu mm are reached by 5 to 7 days and remain below this level or completely disappear before death 7 to 21 days after exposure

The Susceptibility to Infection Its Relation to Mortality and the Granulocyte Count

Warren and Whipple[45] and Lawrence and Tennant[46] working with high doses of radiation that produced death in a few days concluded that infection is not an important factor in roentgen ray deaths This conclusion was confirmed by Osborne *et al* [4] in mice with large doses (2000–3000 r) Gonshery *et al* [48]

Th d p lm t m m l t th d f th d g Aft 3000 m l m d th l f th d y bef m mum d p h d Aft 10 000 m re d g d bef l k p h tum t d l p

TABLE 3

TIME OF APPEARANCE OF MAXIMUM DEPRESSION AND RECOVERY TO NORMAL IN GRANULOCYTES FOR VARIOUS SPECIES AND REPRESENTATIVE DOSES OF X RAY

Species	Dose in r	Per cent mortality	Time to maximum depression in days	Time to recovery to normal in days
Rat	500	50	5	20
	00	—	4	—
	500	—	3–4	25
	550	50	3–5	16–20
	6 0	68	2	—
	800	90	4	30–40
	800–1500	100	3	—
Mouse	600	—	4–6	26–30
LAf1	650	—	4–6	26–30
	800	80	5	20–30
Dog	150	0	14–16	30
	300	50	13 & 19	>30
	400	88	16	>30
	600	100	5–10	—
Monkey	528	50	6–10	Most by 26 days some 41 days
Cat	200	—	3–4	40–50
Hamster	875	90	5–7	15
Swine	400	12	6 & 21	60–90
	600	87	5	60–120
Man	?	50?	5–10 ? & 24	—

Dat r sembl d f m f e s 9 10 11 27 30 33 35 36 37 38 39 40 41 4 43 44

The granular leukocytosis* consisted of two peaks one at 8 to 12 hours and a later one at 24 hours Thereafter there was a decrease The magnitude of the depression and within limits of the duration was a function of the dose of radiation This primary depression may be followed by an abortive rise and a secondary depression clearly described in the rabbit by Jacobson *et al*[9] In TABLE 3 are tabulated the time to maximum depression and time for recovery to normal for the granulocytes in various species after various doses of radiation There is relatively little difference in the rate of development of the primary granulocytopenia for comparable doses of radiation However at the same per cent mortality the granulocytopenia develops more slowly in the dog There are marked species differences in the rate of recovery The hamster recovers most rapidly and man and swine most slowly In FIGURE 2 the relative rates of depression and recovery in the granulocytes are plotted for the hamster and the dog at an approximate LD_{90} for both species The human data are from a group that approximates an LD_{50} [40] The very rapid recovery of granulocytes in the hamster† is evident The intermediate recovery rate in the dog and the very sluggish recovery in man is apparent Since the mortality rates for the three species are comparable in the present instance it is apparent that survival is compatible with different duration of granulocytopenia in different species A general correlation exists however

S ce leth l d se p llel se t ty t bo m w d t lym l od ll t l lym locyt re m it d f m th a t l

† Ham t d ta k dly ppl ed by Doct W W Sm th U t d Stat N t I I t t t f H lth

TEMPERATURE °F (RECTAL)

600A DEATHS

600A

400A

400A DEATHS

DAYS AFTER IRRADIATION

Fig. 4 [illegible] between [illegible] dose [illegible] set of [illegible] mortality. Infections develop [illegible] 400 [illegible]

The most convincing evidence in support of infection as a major lethal factor is the quantitative study of Miller *et al*[51]. These investigators cultured the blood and spleen of serially sacrificed mice at daily intervals following various doses of radiation. The daily per cent mortality and the per cent of positive blood and spleen cultures increased in parallel to a peak on the 10th day and thereafter progressively decreased together, a correlation that has been confirmed by others[45, 5]

If infection were the only lethal factor in radiation injury in the lethal range, germ free animals would not succumb to radiation after doses in this range. Reyniers *et al*[52] have studied this problem in germ free rats. The maintenance of the germ free state alone, while irradiating and studying the animals, is a formidable task and only a limited number of animals were exposed. However, their studies have demonstrated conclusively that animals still die though with a longer survival time. Deaths are apparently due to hemorrhage and anemia. The LD_{50} probably will be moderately increased.

Since infection followed by septicemia and death had been observed early in the radiation era, studies on antibody production were not long delayed. An early study was that of Hektoen[54] in which it was demonstrated that irradiation prevented antibody production to a great extent when the antigen was administered after irradiation, but little, if at all, when irradiation followed antigen administration at the time of maximal antibody production. Many studies[55, 60] have proved that irradiated animals are poor antibody producers

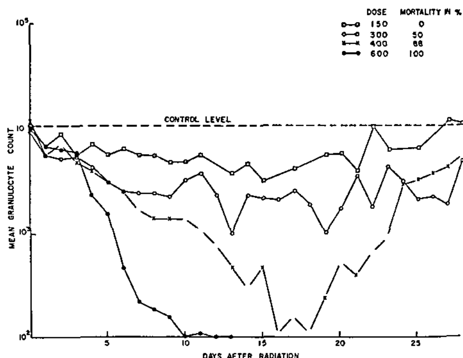


FIGURE 3 G l cyte level n d g e posed to s bl thal a d l th l doses of i d t Note d s de pend ncy of slope f f ll g locyt

came to the same conclusions in respect to the 3 and 4-day deaths of mice after 1100 and 1400 r

Osborne[47] and Silverman *et al* [60] were unable to increase the survival time or decrease the mortality of the GI syndrome by antibiotics even though Silver man *et al* were able to reduce significantly the incidence of bacteremia by the use of streptomycin in mice exposed to sufficient irradiation to produce the GI syndrome It appears that infection is only incidental in the GI syndrome

After lower doses of radiation when animals live longer Chrom[49] and Lawrence and Tennant[46] came to the conclusion that infection is much more prevalent and is a major lethal factor This fact was also apparent in many of the Japanese dying 2 to 4 weeks after the bombing [27, 40] Infectious proc esses were observed over the entire lethal range in animals exposed to the atomic bomb [34] In dogs [21] deaths with obvious clinical infection manifested by cellulitis of the lips neck and legs oropharyngeal and anal ulceration and pneumonias were seen The development of fever was studied and it appeared to be a consistent precursor of death in dogs exposed to 400 and 600 r as illus- trated in FIGURE 4 Following exposure to 600 r the temperature remained within the normal range for the first 5 to 6 days and thereafter the mean temperature of the group increased progressively to about 106° F Deaths with infections listed above occurred between the 6th and 13th days After 400 r, an approximate LD_{90} for dogs the onset of the temperature increase is later and more gradual Deaths occurred between the 13th and 23rd days

sistant bacteria invaded In other cases hemorrhage and anemia may have caused death before adequate recovery of hemopoiesis occurred It appears certain from the studies of Smith *et al*[43,66,67,65] that antibiotics prolong life during the period that the granulocytes are below the critical level for preven tion of spontaneous infections thus allowing time for the recovery of hemopoiesis Various postirradiation protective measures support this concept For example preirradiation spleen shielding postirradiation implantation of spleens the injection of bone marrow or splenic homogenate parabiosis and preirradiation treatment with sulfhydryl compounds have been shown to in crease the survival rate of animals and to accelerate the regeneration of all hemopoietic tissues[81, 4-81]

Congdon *et al*[82] have shown that masses of bacteria that are ordinarily ob erved in mice dying from irradiation are absent when the irradiation is fol lowed by intravenous injection of bone marrow Marston *et al*[83] have demon strated that the administration of splenic homogenates increases the resistance of irradiated mice to experimental infection and complements the action of streptomycin Kaplan *et al*[84] have found the survival time of mice inoculated with the beta hemolytic streptococcus after irradiation to parallel in general the leukocyte curve Using splenic homogenates induced bacterial infection and spontaneous infection Smith *et al*[77, 65] have shown that the probability of death is correlated with the granulocyte count and is independent of the lymphocyte count In their first study[77] sublethally irradiated mice were challenged by the injection of bacteria and a very definite association between resistance to *Pseudomonas aeruginosa* infection and the granulocyte count was determined In general there was an increase in survival of approximately 5 per cent for every 100 granulocytes in the animals that were challenged by bacterial injection after an otherwise sublethal irradiation To quote Thus within the limitation imposed by our procedures general recovery whether occurring naturally or accelerated by spleen homogenate adds to survivorship relatively little that is not measurable as a function of the granulocyte count There was no correlation with the lymphocyte count Similar studies[65] were made on animals irradiated with a dose of 550 r spontaneous mortality of 65 per cent When the granulocytes were ranked according to increasing granulocyte levels in six groups there was a progressive increase in survival from 21 to 64 per cent There was no correlation between lymphocyte level and survival rate These systematic and well controlled studies demonstrate for the mouse that chance of survival following induced or spontaneous infection is propor tional to the granulocyte count but do not claim to prove that other subtle factors are not as important

All of the preceding studies imply that there are critical levels of the granulocyte below which survival is improbable and above which survival is probable The range in granulocytes over which survival is proportional to the count has been quantified only in the mouse[77, 65]

From closer inspection of our data[56] on dogs it appears that the upper level above which survival can generally be expected is in the vicinity of 1200 granu locytes per cu mm and that survival is rare when granulocytes remain below 500 per cu mm for any length of time

The ability to make antibodies varies with dose of radiation time after irradiation time and dose of antigen and the species However the rate of disappearance of preformed antibody is not impaired[61] and the rate of disappearance of passively transferred antibody is unchanged [6]

Clearly, there are major impairments in the ability to produce antibodies However, evidence that the susceptibility to infection of the irradiated animal is well correlated with antibody production is lacking In our own and other studies it appeared that the susceptibility to infection was well correlated with the granulocyte The absolute level of the granulocytes the rate at which the count fell and the duration of depression seemed of major importance These factors were evident in studies with dogs [63] At a 10 per cent mortality there was a slow progression in the development of the granulocytopenia with minimum levels of 40 per cent of normal appearing by the 16th day As the mortality increased the rate at which the granulocytes fell increased and the depth to which the granulocytes fell also increased At 80 per cent mortality the fall was more rapid and by the 13-14th day, there were less than 1000 cells present At 100 per cent mortality the cells had virtually disappeared from the blood by the fifth day and deaths occurred rapidly These features are also illustrated in FIGURE 3 In FIGURE 4 it is seen that the temperature increases more slowly in the 400 r group when leukopenia develops more gradually than in the 600 r group A more detailed review of the pathology and pathogenesis of infection in the irradiated animal has been given by Bond *et al* [6]

If bacterial infection is the major cause of death in the lethal range for most mammals, antibiotics should significantly increase the survival time and the survival rates This would be true however only provided that recovery of other phases of hemopoiesis namely the production of thrombocytes and erythrocytes took place with sufficient rapidity to prevent death from hemorrhage and anemia Miller *et al* [65] have conclusively shown that provided that resistant bacteria are not present streptomycin will increase the survival time and survival rate of mice within the lethal range but not from supralethal doses of radiation This work has been confirmed in mice by other investigators [45, 66, 67] Antibiotics have been of definite value in hamsters [68] swine [69] dogs [70] and rats [71] A convincing demonstration of the value of antibiotics in the irradiated animal was that of Baxter *et al* [69] in which it was shown that 400 r produced only a 20 per cent mortality in swine but when a 10 to 15 per cent flash burn was superimposed upon this irradiation providing an open wound for the entry of bacteria the mortality increased to 90 per cent The use of streptomycin decreased the mortality to that of irradiation alone Koletzky[71] had good results in rats given internal irradiation by combining penicillin and streptomycin A striking demonstration of the value of using combined and successive antibiotics is that of Coulter and Miller[7] in dogs Whereas previous studies in dogs and rats with single antibiotics had yielded equivocal results the use of multiple antibiotics in succession gave a significant improvement in survival rate

The reasons for the failure of antibiotics in some experiments are not entirely clear In some cases the antibiotics may have been ineffectual because re

relationship between life span and rate of disappearance of cells obtains only when cessation of production is complete or nearly complete If production does not cease completely, and the remaining precursor cells continue to divide in a normal fashion the speed of the decrease of the leukocyte count is slowed down by the addition of new cells until a new dynamic equilibrium is established at a lower level The possibility also exists that partial destruction of precursor cells is associated with functional abnormalities of the surviving pre cursor cells resulting in changing production rates For example, in the dog (FIGURE 3) 150, 300 400 and 600 r produce a progressive decline in the granu locytes over many days but at different rates Since the slopes are different one has to assume changes in the life span, or a continuously decreasing production rate or other aberrations such as altered utilization that varies with the defense and metabolic needs of the animal In cases where there is a pri mary and secondary depression separated by an abortive rise it is logical to assume that a temporary increase in production rate precedes the abortive rise Bloom s observation[8] of a temporary wave of hemopoietic activity in parallel with the abortive rise supports the supposition of varying production rates Further experimental work is needed to assess to what extent radiation induced alterations of life span and utilization contribute to the shape of the observed leukocyte curves

There is no doubt that the primary cause of the postirradiation pancytopenia is the marrow hypoplasia The latter is produced by mitotic injury to pre cursor cells On the basis of numerous studies[96 100] on mitotic systems that are more easily observed than hemopoietic tissue it can be inferred that the degree and to an extent the type of mitotic injury will vary with the dose of radiation If the level of radiation is sufficiently high all cells in the premitotic and mitotic stage are damaged to the extent that mitosis does not continue In the particular case there is from the time of exposure a progressive linear regression from the preirradiation count At lower doses of irradiation mi tosis is inhibited for varying lengths of time in those cells that are in the pre mitotic phase The cells that are in mitosis may continue to divide and their daughter cells may undergo one or more divisions Ultimately these daughter cells may die Regeneration takes place either from those cells that were in the premitotic stage at the time of irradiation or begins again by differentiation and division of the primitive mesenchymal tissue By indirect study of autopsy material in Japan[40] it appeared that some of the regeneration was occurring at such a primitive level

Modification of Leukopenia

Sterile inflammation induced by turpentine injections intramuscularly has long been known to produce a stimulation of granulocytopoiesis manifested by a peripheral granulocytosis a shift to the left in the differential count and a hyperplasia of myeloid elements It has been reported[100] that turpentine spe cifically decreases the intermitotic time of the myeloid series If the primary defect leading to bone marrow aplasia is a prolongation of the intermitotic time of the myeloid series a natural stimulus such as sterile inflammation might stimulate the return of granulocytopoiesis If the concepts previously dis-

Indirect evidence that emphasizes the critical role of the bone marrow in survival is the fact that the LD_{50} is dependent upon the regenerative ability of the bone marrow[71, 87] and is independent of the sensitivity of the lymphoid tissue[21, 87, 88] Since it has been shown[63, 89, 90] that maintenance of red cell and platelet levels does not significantly increase the survival rate of irradiated animals it is logical to consider that the continued production or the resumption of production of granulocytes are primarily responsible for spontaneous survival from irradiation in the lethal range

The evidence presented would appear to establish firmly a correlation between granulocytopenia and susceptibility to infection in radiation injury It has been questioned, however, whether a depression of the granulocyte level is the constant and sole cause of mortality from infection[91, 92] Shechmeister *et al*[91] found that the maximum susceptibility of sublethally irradiated mice to infection occurred 15 days after exposure and were unable to make a positive correlation of the increased susceptibility with levels of white cells or ability to produce antibodies In addition, it has been shown by Shechmeister and Fishman[93] that the migration of rabbit and rat leukocytes is depressed at certain intervals after exposure The depression in migratory ability corresponds with the primary and secondary depression in the granulocyte count During the "abortive rise" the migratory ability returns to normal It was shown that the depressed migration was not due to a plasma factor In addition the bactericidal effect of leukocyte extracts from irradiated rats was found to be depressed at three days after irradiation[94] However, in the preceding studies[93, 94] the influence of a changing and aging population of leukocytes on the migratory and bactericidal activity of leukocytes was not investigated

Mechanisms Involved in the Production of Leukopenia and Recovery Therefrom

Under normal conditions the formed elements of the blood are in a dynamic equilibrium Production equals utilization The diurnal nocturnal variations in the leukocyte levels are believed to represent surges of production release from the bone marrow or adjustment of distribution under various physiologic stimuli superimposed upon an essentially constant level for the species or individual If production of new cells ceased completely without change in their normal life span the leukocyte level would decrease in the peripheral blood as a function of the life span of the various types of leukocytes Under these conditions, the speed with which the number of leukocytes decreases would be a function of the life span The situation would be analogous to determination of red cell survival by the Ashby technique where the slope of the linear regression of the cell population is a function of the life span *

These problems have been approached experimentally by Lawrence *et al*[10] and Valentine[44, 95] In rats after 500 r, approximately an LD_{50} depression of granulocytes was maximal in 72 hours The actual rate of disappearance closely approximates the rate expected from estimates of the life span of leukocytes in previous cross transfusion experiments However such a simple

S th re d th t le kocyt p freely d t f th s l bed th l f p sod t rm d would clud th t tal t cula d t a s lar l f f th cells

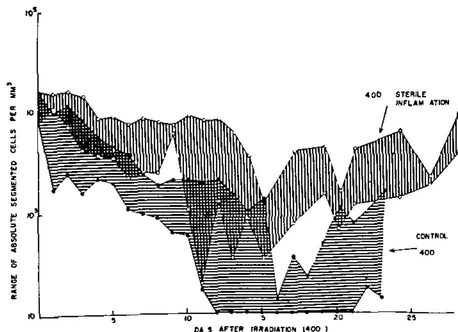
suggest that the inflammatory response produced by the intraperitoneal injection of ground bone may be responsible for the stimulation of hematopoiesis The recent independent studies of Smith *et al*,[103] in which ground glass and other inert particulate material was injected intraperitoneally in mice after irradiation showed a significant increase in survival rate In these studies a humoral factor cannot be implicated and the presence of an inflammatory response of the peritoneum can be assumed

In other studies[104] talc portland cement and ground heterologous bone with and without turpentine was injected intraperitoneally into rats and guinea pigs after irradiation with graded doses The survival rate below an LD_{50} was slightly increased even though many animals died at an early period from intestinal obstruction and peritoneal abscesses Bone marrow smears suggested that there was an earlier return of hematopoiesis In addition Farr[105] has shown that the intravenous injection of pyrogens (killed typhoid bacilli) will significantly alter the granulocyte response of rabbits to irradiation The counts in the treated animals remain higher than the counts in the control animals Comparable results have been obtained by Strumia *et al*[106] by injecting human and rabbit leukocytic extracts into irradiated rabbits Ingesting of purified pyrogens by irradiated mice also altered the leukocyte count in a similar manner[107] In the studies of Farr and Strumia the survival rate was not increased

In contrast to the beneficial results of induced inflammation in rats and guinea pigs an increased mortality rate was observed over the entire lethal range in a series of experiments in which talc ground bone and turpentine were injected into mice[104] Storer reported the failure of the leukocytosis promoting factor of Menkin to improve the survival rate in mice[108] In some cases the detrimental effect of the inflammatory response was explained by the severe peritonitis peritoneal abscesses and intestinal obstruction In other instances the detrimental effect may be due to actual damage to marrow cells by early stimulation Studies of fertilization in sea urchin eggs[109] and bacteria[110] indicate that a longer rest period is needed after higher doses of irradiation before cell division can be induced without causing further cell deaths Similarly a longer rest period may be needed before hematopoiesis can be stimulated by induced inflammation This necessity may explain the failure to obtain a beneficial effect above an LD_{90} with early stimulation However at these higher doses the granulocytes disappear promptly and it is not possible to induce an inflammatory stimulus of the bone marrow The separation and concentration of leukocytes should now permit the induction of sterile inflammatory reactions at any time interval after irradiation thus making it possible to determine if the products of granulocytic exudation can significantly stimulate regeneration of granulocytopoiesis in bone marrow damaged by irradiation or other causes

To date our own data and those of others suggest strongly that the granulocyte level in the peripheral blood and to a limited extent the survival rate of animals can be increased by postirradiation treatment under conditions that eliminate the possibility of introduction of an exogenous humoral stimulus or the transplantation of viable hemopoietic precursors

cussed, of the factors leading to infection and death are correct, an early return of granulocytopoiesis should significantly increase the survival rate in the lethal dose range, as already suggested by the reduced mortality rate of animals injected with bone marrow or spleen homogenates The work of Schlang[101] on the stimulation of bone marrow in nitrogen mustard treated rabbits provided the impetus for experiments along these lines [10] Injection of dogs with sterile turpentine, dead bacteria, and bacterial filtrates immediately after irradiation or during the time that granulocytes remained above 1500/cu mm resulted in a prompt inflammatory response, often with the development of a fluctuant abscess In dogs that had received 400 r an LD_{90}, a stimulation of granulocytopoiesis was obtained as indicated by differences in the granulocyte counts of the treated and untreated dogs, illustrated in FIGURE 5, in which the ranges in absolute granulocyte counts of treated and control dogs are plotted There is some overlapping during the first seven days and between the 10th and 15th days The somewhat earlier recovery of the granulocyte count is evident There were no differences in the degree of thrombopenia or anemia between the groups In these preliminary studies the differences in survival rate are encouraging In treated dogs the survival rate was increased from 17 per cent in the 18 controls to 50 per cent in the 18 treated animals Lorenz and Congdon[81] injected fragments of bone and ground homologous and heterologous bone intraperitoneally into mice after irradiation The survival rate was significantly increased Lorenz and Congdon interpret their data as evidence in support of a humoral factor in the bone that stimulates hematopoiesis We



F GURE 5 R ge of gr l cyte count f d g wh h t le flamm t w d d ft d t n w th 400 r mp d w th g a l cyt t of tr l d g posed t 400

TABLE 4

TRANSFUSION OF SEPARATED WBC INTO A DOG IRRADIATED WITH 600 r

Day after X-ray	Leukocytes $\times 10^8$ injected	Circulating leukocytes $\times 10^3$ per cu mm		
		Before transfusion	1 hr after transfusion	4-6 hrs after transfusion
5	102	8	18	
6	74	6	28	49
7	47	11	27	41
8	52	10	22	44
9	73	12	32	42
10	77	3	19	
11		17	16	
12	46	9	12	
13	1.6	3	18	
14	5	4	8	
15	59	7	6	4
16	62	2	6	4
17	52	1	4	2
18		0.2	3	
19	59	1	4	4
20	69	1	2	3

Conclusions

(1) There are species differences in response of granulocytes to irradiation

(2) Infection is a major cause of death in the lethal range for mammals

(3) Susceptibility to infection is best correlated with the lowered resistance produced by the granulocytopenia

(4) There are various mechanisms by which the granulocytopenia and mortality can be altered

(5) Leukocytes can be separated concentrated and transfused into homologous recipients

(6) Transfused leukocytes migrate to sites of infection

Bibliography

1 HEINECKE H 1903 Ueber die Einwirkung der Roentgenstrahlen auf Tiere Münch med Wochschr 1 2090-2092

2 HEINECKE H 1904 Ueber die Einwirkung der Roentgenstrahlen auf innere Organe Münch med Wochschr 51 785

3 HEINECKE H 1905 Experimentelle Untersuchungen ueber die Einwirkung der Roentgenstrahlen auf das Knochenmark nebst einigen Bemerkungen ueber die Roentgentherapie der Leukemie und Pseudoleukaemie und des Sarkoms Deut Z Chir 78 196-230

4 MINOT C R & R G SPURLING 1924 The effect on the blood of irradiation especially short wave length roentgen ray therapy Am J Med Sci 168 215-241

5 TAYLOR H D W D WITHERBEE & J B MURPHY 1919 Studies on X ray effects Destructive action on blood cells J Exptl Med 29 53-73

6 WARREN S 1942 The effects of radiation on normal tissue Arch Path. 34 443 562 749 917 10 0

7 SELLING L & E F OSGOOD 1938 Action of benzol roentgen rays and radioactive substances on the blood and blood forming tissues Handbook of Hematology H Downey Ed Hoeber New York N Y

8 BLOOM W 1948 Histopathology of Irradiation from External and Internal Sources McGraw Hill New York N Y

Separation and Transfusion of Leukocytes

The first method for the separation of leukocytes was based on the fact that injection of turpentine produced an abscess a leukocytosis, and an increased plasma fibrinogen level The latter accelerates the sedimentation of red cells leaving platelets and leukocytes in suspension With this method, leukocyte platelet concentrates were prepared, transfused, and circulated in the pancytopenic dog However, the method is not practical on a large scale Dextran, another large asymmetric molecule, can be substituted for fibrinogen Certain dextrans had been used by McKinney *et al* [111] to separate leukocytes on a small scale The leukocytes so separated, appeared to be metabolically intact as measured by rates of oxygen consumption and glycolysis Details of the larger scale separation of leukocytes by the dextran method have been published [112] With 500 to 600 milliliters of blood with leukocyte levels of 10 000 to 15 000/cu mm suspensions of leukocytes containing approximately 5×10^9 leukocytes have been obtained with fair consistency The red cell contamination is small, except when the donor dogs are anemic with high levels of reticulocytes, which are not sedimented by dextran

To date there have been no acute reactions to the initial transfusion of the leukocyte platelet concentrate After repeated transfusions with the leukocyte platelet suspensions some urticarial reactions with pulmonary symptoms have been seen The precise cause of these reactions has not been ascertained They may be due to sensitization to the homologous leukocytes or platelets There has been one unquestioned case of sensitization to canine platelets [113]

Following the infusion of leukocyte suspensions into leukopenic dogs it is apparent that the level of the transfused leukocytes does not promptly rise to a maximum and then decrease as a function of their life span The four hour concentration is greater than the one hour concentration in venous blood An example of the transfusion of leukocytes is shown in TABLE 4 After the 13th day very few leukocytes circulated Whether this effect was due to isoimmunization was not determined

The transfused leukocytes have circulated in several dogs Pathologic studies[114] on sacrificed animals have shown that the transfused leukocytes will migrate to sites of infection and produce an inflammatory exudate So far leukocytes have been found in impetiginous lesions of the skin hair follicles (folliculitis) ulcerated tonsils and lymph nodes of dogs that have total aplasia of the bone marrow

Whether the replacement of leukocytes will increase the survival rate has not been determined as yet Superficially it appears that replacement therapy would be highly impractical because of the short life span of leukocytes However from the work of Smith[77, 85] and ourselves it appears probable that it will not be necessary to maintain a very high leukocyte level in order to increase significantly the survival rate provided that the transfused leukocytes are functionally adequate

The use of separated leukocytes in conjunction with inflammatory agents to incite inflammation and stimulate myelopoiesis in animals with hypoplastic marrows is under study

TABLE 4
TRANSFUSION OF SEPARATED WBC INTO A DOG IRRADIATED WITH 600 r

Day after X-ray	Leukocytes × 10^3	Circulating leukocytes × 10 mm		
		Before transfusion	1 hr after transfusion	4-6 hrs after transfusion
5	102	8	18	
6	74	6	28	49
7	47	11	22	41
8	52	10	22	44
9	73	12	32	4[illegible]
10	[illegible]7	3	19	
11		12	16	
12	46	9	12	
13	126	3	18	
14	57	4	8	
15	59	7	6	4
16	6[illegible]	2	6	4
17	52	1	4	2
18		0 2	3	
19	59	1	4	4
20	69	1	2	3

Conclusions

(1) There are species differences in response of granulocytes to irradiation

(2) Infection is a major cause of death in the lethal range for mammals

(3) Susceptibility to infection is best correlated with the lowered resistance produced by the granulocytopenia

(4) There are various mechanisms by which the granulocytopenia and mortality can be altered

(5) Leukocytes can be separated concentrated and transfused into homologous recipients

(6) Transfused leukocytes migrate to sites of infection

Bibliography

1 HEINECKE H 1903 Ueber die Einwirkung der Roentgenstrahlen auf Tiere Münch med Wochschr 1 2090–2092
2 HEINECKE H 1904 Ueber die Einwirkung der Roentgenstrahlen auf innere Organe Munch med Wochschr 51 785
3 HEINECKE H 1905 Experimentelle Untersuchungen ueber die Einwirkung der Roentgenstrahlen auf das Knochenmark nebst einigen Bemerkungen ueber die Roentgenthe apie der Leukemie und Pseudoleukaemie und des Sarkoms Deut Z Chir 78 196–230
4 MINOT G R & R G SPURLING 1924 The effect on the blood of irradiation especially short wave length roentgen ray therapy Am J Med Sci 168 215–241
5 TAYLOR H D W D WITHERBEE & J B MURPHY 1919 Studies on X ray effects Destructive action on blood cells J Exptl Med 29 53–73
6 WARREN S 1942 The effects of radiation on normal tissue Arch Path 34 443 56? 749 917 10 0
7 SELLING L & E E OSGOOD 1938 Action of benzol roentgen rays and radioactive substances on the blood and blood forming tissues Handbook of Hematology H Downey Ed Hoeber New York N Y
8 BLOOM W 1948 Histopathology of Irradiation from External and Internal Sources McGraw Hill New York N Y

Separation and Transfusion of Leukocytes

The first method for the separation of leukocytes was based on the fact that injection of turpentine produced an abscess a leukocytosis, and an increased plasma fibrinogen level The latter accelerates the sedimentation of red cells leaving platelets and leukocytes in suspension With this method leukocyte platelet concentrates were prepared transfused and circulated in the pancytopenic dog However, the method is not practical on a large scale Dextran another large asymmetric molecule, can be substituted for fibrinogen Certain dextrans had been used by McKinney *et al* [111] to separate leukocytes on a small scale The leukocytes, so separated appeared to be metabolically intact as measured by rates of oxygen consumption and glycolysis Details of the larger scale separation of leukocytes by the dextran method have been published [112] With 500 to 600 milliliters of blood with leukocyte levels of 10 000 to 15,000/cu mm, suspensions of leukocytes containing approximately 5×10^9 leukocytes have been obtained with fair consistency The red cell contamination is small, except when the donor dogs are anemic with high levels of reticulocytes, which are not sedimented by dextran

To date there have been no acute reactions to the initial transfusion of the leukocyte platelet concentrate After repeated transfusions with the leukocyte platelet suspensions some urticarial reactions with pulmonary symptoms have been seen The precise cause of these reactions has not been ascertained They may be due to sensitization to the homologous leukocytes or platelets There has been one unquestioned case of sensitization to canine platelets [113]

Following the infusion of leukocyte suspensions into leukopenic dogs it is apparent that the level of the transfused leukocytes does not promptly rise to a maximum and then decrease as a function of their life span The four hour concentration is greater than the one hour concentration in venous blood An example of the transfusion of leukocytes is shown in TABLE 4 After the 13th day very few leukocytes circulated Whether this effect was due to isoimmunization was not determined

The transfused leukocytes have circulated in several dogs Pathologic studies[114] on sacrificed animals have shown that the transfused leukocytes will migrate to sites of infection and produce an inflammatory exudate So far leukocytes have been found in impetiginous lesions of the skin hair follicles (folliculitis) ulcerated tonsils and lymph nodes of dogs that have total aplasia of the bone marrow

Whether the replacement of leukocytes will increase the survival rate has not been determined as yet Superficially it appears that replacement therapy would be highly impractical because of the short life span of leukocytes However from the work of Smith[77, 85] and ourselves it appears probable that it will not be necessary to maintain a very high leukocyte level in order to increase significantly the survival rate provided that the transfused leukocytes are functionally adequate

The use of separated leukocytes in conjunction with inflammatory agents to incite inflammation and stimulate myelopoiesis in animals with hypoplastic marrows is under study

TABLE 4

TRANSFUSION OF SEPARATED WBC INTO A DOG IRRADIATED WITH 600 r

Day after X-ray	Leukocytes × 10⁹ inj	Circulating leukocytes × 10³/mm³		
		Before transfusion	1 hr after transfusion	4-6 hr after transfusion
5	102	8	18	
6	74	6	28	49
7	47	11	2[illegible]	41
8	52	10	2[illegible]	44
9	73	12	32	42
10	7	3	19	
11		12	16	
12	46	9	12	
13	126	3	18	
14	57	4	8	
15	59	7	6	4
16	62	2	6	4
17	52	1	4	2
18		0 2	3	
19	59	1	4	4
20	69	1	2	3

Conclusions

(1) There are species differences in response of granulocytes to irradiation

(2) Infection is a major cause of death in the lethal range for mammals

(3) Susceptibility to infection is best correlated with the lowered resistance produced by the granulocytopenia

(4) There are various mechanisms by which the granulocytopenia and mortality can be altered

(5) Leukocyte can be separated concentrated and transfused into homologous recipients

(6) Transfused leukocytes migrate to sites of infection

Bibliography

1 HEINECKE H 1903 Ueber die Einwirkung der Roentgenstrahlen auf Tiere Münch. med Wochschr 1 2090–2092

2 HEINECKE H 1904 Ueber die Einwirkung der Roentgenstrahlen auf innere Organe Münch med Wochschr 61 185

3 HEINECKE H 1905 Experimentelle Untersuchungen ueber die Einwirkung der Roentgenstrahlen auf das Knochenmark nebst ein gen Bemerkungen ueber d e Roentgentheraple der Leukemie und Pseudoleukaemie und des Sa koms Deut Z Chir 78 196–230

4 MINOT C F & R G SPURLING 1924 The effect on the blood of irradiation especially short wa e length roentgen ray therapy Am J Med Sci 168 215–241

5 TAYLOR H D W D WITHERBEE & J B MURPHY 1919 Studies on X ray effects Destructive action n blood cells J Exptl Med 29 53–73

6 WARREN S 1942 The effects of radiation on normal tissue Arch Path 34 443 562 749 917 1070

7 SELLING L & E F OSGOOD 1938 Action of benzol roentgen rays and radioactive substances on the blood and blood forming tissues Handbook of Hematology H Downey Ed Hoeber New York N Y

8 BLOOM W 1948 Histopathology of Irradiation from External and Internal Sources McGraw Hill New York N Y

9 Jacobson L O E K Marks & E Lorenz 1949 Hematological effects of ionizing radiations Radiology 53 371-395
10 Lawrence J S A H Dowdy & W N Valentine 1948 Effect of radiation on hemopoiesis Radiology 51 400-413
11 Behrens C F 1953 Atomic Medicine Chaps 8 & 10 Williams & Wilkins Baltimore Md
12 Cronkite E P & G Brecher 1952 Radiology and radioactivity Effects of whole body irradiation Annual Review of Medicine Stanford Univ Press Stanford Cal 3 193-214
13 Patt H M 1953 Protective mechanisms in ionizing radiation injury Physiol Revs 33 35-76
14 Patt H M 1954 Radiation effects on mammalian systems Ann Rev Physiol 16 51-80
15 Furth J & A C Upton 1954 Vertebrate radiobiology Carcinogenesis and histopathology Ann Rev Nuclear Sci 3 303-338
16 Ellinger F 1941 Biologic Fundamentals of Radiation Therapy Elsevier Pub Co New York N Y
17 Rajewsky B & A Schraub Strahlenschaedigungen Allgemeine Schaedigungskurven V Rajensky & M Schoen Biophysics 1 173-131
18 Cronkite E P 1951 Diagnosis prognosis and treatment of radiation injuries produced by atomic bombs Radiology 56 661-668
19 Langham W S Rothermel K T Woodward C C Lushbaugh J B Storer & P S Harris 1954 Studies of the effects of massive rapid doses of gamma rays on mammals Los Alamos Sci Lab Report LA 1643
20 Andrews H L & K C Brace 1953 Terminal phenomena associated with massive doses of X ray in guinea pigs Am J Physiol 175 138-140
21 Brecher, G & E P Cronkite Pathologic analysis of lethal factors in radiation injury To be published
22 Williams R B Jr & R P DeLong 1953 Effect of spleen shielding glutathione and para aminopropiophenone upon recovery of the small bowel epithelium of the rat following whole body X radiation Federation Proc 12 406
23 Quastler H E F Lanzl M E Keller & J W Osborne 1951 Acute intestinal radiation death Studies on roentgen death in mice Am J Physiol 164 546-556
24 Bond V P M N Swift A C Allen & M C Fishler 1950 Sensitivity of abdomen of rat to X irradiation Am J Physiol 161 323-330
25 Brecher G & E P Cronkite 1951 Lesions of the alimentary tract of dogs exposed to 300-3000 r of total body X irradiation Am J Path 27 676-677
26 Bond V P M S Silverman & E P Cronkite 1954 Pathogenesis and pathology of post irradiation infection To be published in Radiation Research
27 LeRoy G V 1947 Medical sequelae of the atomic bomb explosion J Am Med Assoc 134 1143-1148
28 Williams R B Jr 1954 Tissue and species differences in the duration of mitotic *inhibition on prolongation of interphase by X radiation Federation Proc 13 446*
29 Tullis J L F W Chambers Jr J E Morgan & J H Zeller 1952 Mortality in swine and dose distribution studies in phantoms exposed to supervoltage roentgen radiation Am J Roentgenol 67 620-627
30 Patterson E 1954 Factors influencing recovery after whole body irradiation J Faculty Radiologists 5 189-199
31 Brecher G & E P Cronkite 1951 Post radiation parabiosis and survival in rats Proc Soc Exptl Biol Med 77 292-294
32 Smith W W Personal communication
33 Ingram M & W B Mason 1950 The effects of acute exposure to roentgen radiation *on the peripheral blood of experimental animals Univ Rochester UR 122*
34 Tullis J L 1949 The response of tissues to total body irradiation Am J Path 25 829-851
35 Cronkite E P 1949 Clinical manifestations of acute ionizing radiation illness in goats exposed to an atomic bomb explosion U S Naval Med Bull 49 199-215
36 Cronkite E P 1950 The hemorrhagic syndrome of acute ionizing radiation illness produced in goats and swine by exposure to the atomic bomb explosions at Bikini Blood 5 32-45
37 Ting T P H E Johns & L B Jaques 1952 The relative biologic effectiveness of 200 KV and 23 5 MEV (Betatron) X radiation I Production of leukopenia in mice Blood 7 826-835
38 LeRoy G V 1950 Hematology of atomic bomb casualties Arch Internal Med 86 691-710

39 ELDRED E & B ELDRED 1953 Effects of total body X irradiation on the peripheral blood of the monkey Blood 8 262 270
40 Medical Report of Joint Commission for the Investigation of the Effects of the Atomic Bomb in Japan 1947 Vol 5 AEC Document NP-3040
41 PATT H M D E SMITH & E JACKSON 1950 The effect of apteine on the peripheral blood of the irradiated rat Blood 5 758–763
42 STORER J B C C LUSHBAUGH & J E FURCHNER 1952 The protective effect of shielded ectopic bone marrow against total body X radiation J Lab Clin Med 40 355–366
43 CRONKITE E P F W ULLRICH D C ELTZHOLTZ C R SIPE & P K SCHORK 1949 The response of the peripheral blood of swine to whole body X ray radiation in the lethal range Navy Med Research Inst Rpt Project NM 007 039 Report No 21 Bethesda Md
44 VALENTINE W N & M L PEARCE 1952 Studies on the radiosensitivity of bone marrow I The relative sensitivity of erythroid and myeloid elements Blood 7 1–13
45 WARREN S L & G H WHIPPLE 1923 Roentgen ray intoxication Bacterial invasion of the blood stream as influenced by X ray destruction of the mucosal epithelium of the small intestine J Exptl Med 38 713–723
46 LAWRENCE J H & R TENNANT 1937 The comparative effects of neutrons and X rays on the whole body J Exptl Med 66 667–688
47 OSBORNE J S H S BRYAN H QUASTLER & H E RHOADES 1952 X irradiation and bacteremia studies on roentgen death in mice Am J Physiol 170 414–417
48 GONSHERY L R Q MARSTON & W W SMITH 1953 Naturally occurring infections in untreated and streptomycin treated X irradiated mice Am J Physiol 172 359–364
49 CHROM S A 1935 Studies on the effect of roentgen rays upon the intestinal epithelium and upon the reticulo-endothelial cells of the liver and spleen Acta Radiologica 16 641–660
50 SILVERMAN M S & V P BOND Bacteriological studies on animals exposed to neutron radiation To be published
51 MILLER C P C W HAMMOND & M TOMPKINS 1951 The role of infection in radiation injury J Lab Clin Med 38 331–343
52 SILVERMAN M S V P BOND P H CHIN & V GREENMAN 1954 The role of infections in the death of mice exposed to lethal doses of fast neutrons Federation Proc 13 512
53 REYNIERS J A & Associates in LOBUND ONR AEC Report III 1953 LOBUND study on comparative effects of total body irradiation LOBUND Institute Univ Notre Dame Notre Dame Ind
54 HEKTOEN L 1918 Further studies on the effects of the roentgen ray on antibody production J Infectious Diseases 22 28–33
55 TALIAFERRO W H L G TALIAFERRO & E F JANSSEN 1952 The localization of X ray injury to the initial phases of antibody response J Infectious Diseases 91 105–124
56 JACOBSON L O M J ROBSON & E K MARKS 1950 The effect of X radiation on antibody formation Proc Soc Exptl Biol Med 75 145–152
57 CRADDOCK C G JR & J S LAWRENCE 1948 The effect of roentgen irradiation on antibody formation in rabbits J Immunol 60 241–254
58 DIXON F J D W TALMADGE & P H MAURER 1952 Radiosensitive and radioresistant phases in the antibody response J Immunol 68 693–700
59 BURROWS W N G DEUPREE & D E MOORE 1950 The effect of X irradiation on fecal and urinary antibody response J Infectious Diseases 87 169–183
60 TALIAFERRO W H & L G TALIAFERRO 1951 Effects of X rays on immunity a review J Immunol 66 181–212
61 TALIAFERRO W H & L G TALIAFERRO 1950 Effect of X irradiation on hemolysin decline J Infectious Diseases 87 201–209
62 HOLLINGSWORTH J W 1950 Effects of X irradiation on passively transferred antibodies Proc Soc Exptl Biol Med 75 477–479
63 CRONKITE E P & G BRECHER 1952 Defects in hemostasis produced by whole body irradiation Trans 5th Conf Blood Coagulation J E Flynn Josiah Macy Foundation New York N Y
64 MILLER C P C W HAMMOND & M TOMPKINS 1950 Reduction of mortality from X irradiation by treatment with antibiotics Science 111 719–720
65 MILLER C P C W HAMMOND M TOMPKINS & G SHORTER 1952 The treatment

of postradiation infection with antibiotics experimental study on mice J Lab Clin Med 39 462–479
66 SMITH W W F SMITH H J RUTH H Y CANTER & M M GRENAN 1953 Prophylactic antibiotic therapy in X irradiated animals Am J Physiol 172 351–358
67 MARSTON R Q L GONSHERY I M ALDERMAN & W W SMITH 1953 Experimental infection and streptomycin treatment in irradiated mice Am J Physiol 172 365–371
68 SMITH W W Personal communication
69 BAXTER R J A DRUMMOND L G STEPHENS NEWSHAM & R G RANDALL 1953 Reduction of mortality in swine from combined total body radiation and thermal burns by streptomycin Ann Surg 137 450–455
70 COULTER M P & R W MILLER 1953 Treatment with successive antibiotics on dogs exposed to total body X radiation Univ Rochester AEC Project Rept UR 276
71 KOLETSKY S 1950 Effect of antibiotics on mortality from internal radiation Western Reserve Univ AEC Project Rept NYO 1614
72 HOWLAND J W F FURTH L R BENNETT M COULTER & G M McDONNEL 1949 Studies on factors effecting the radiation syndrome I The effect of aureomycin and antibiotics on whole body irradiation Univ Rochester Atomic Energy Project Rept UR 94
73 FURTH F W & M P COULTER 1950 The effect of aureomycin on the radiation syndrome in dog Univ Rochester Atomic Energy Project Rept UR 146
74 JACOBSON L O E L SIMMONS E K MARKS E O GASTON M J ROBSON & J H ELDREDGE 1951 Further studies on recovery from radiation injury J Lab Clin Med 37 683–697
75 LORENZ E C CONGDON & D UPHOFF 1952 Modifications of acute irradiation injury in mice and guinea pigs by bone marrow injections Radiology 58 863–877
76 COLE L J M C FISHLER M E ELLIS & V P BOND 1952 Protection of mice against X irradiation by spleen homogenates administered after exposure Proc Soc Exptl Biol Med 80 112–117
77 *SMITH W W R Q MARSTON H J RUTH & J CORNFIELD 1954 Granulocyte* count resistance to experimental infection and spleen homogenate treatment in mice Am J Physiol 178(2) 288–292
78 PATT H M D E SMITH & E JACKSON 1950 The effect of cysteine on the peripheral blood of the irradiated rat Blood 5 758–763
79 JACOBSEN L O 1952 Evidence for a humoral factor (or factors) concerned in recovery from radiation injury a review Cancer Research 12 315–325
80 CRONKITE E P G BRECHER & W H CHAPMAN 1951 Studies on the mechanism of the protective action of glutathione against whole body radiation Military Surgeon 109 294–307
81 LORENZ E & C C CONGDON 1954 Modification of lethal irradiation injury in mice by injection of homologous or heterologous bone J Natl Cancer Inst 14 955–965
82 CONGDON C C D UPHOFF & E LORENZ 1952 Modification of acute irradiation *injury in mice and guinea pigs by injection of bone marrow A histopathologic study* J Natl Cancer Inst 13 73–107
83 MARSTON R Q H J RUTH & W W SMITH 1953 Role of host defenses in streptomycin treatment of X irradiated mice Proc Soc Exptl Biol Med 83 289–291
84 KAPLAN H S R S SPECK & E JAWETZ 1952 Impairment of antimicrobial defense following total body irradiation of mice J Lab Clin Med 40 682–691
85 SMITH W W L GONSHERY I ALDERMAN & J CORNFIELD 1954 Effect of granulocyte count and litter on survival of irradiated mice Am J Physiol 178 (3) 474–476
86 CRONKITE E P G BRECHER & P K SCHORK To be published
87 *BRECHER G & E P CRONKITE 1952 Correlation of bone marrow injury with mor*tality in different species of irradiated mammals Am J Path 28 551
88 DEBRUYN P P H 1948 The effect of X rays on the lymphatic nodule with reference to the dose and relative sensitivities of different species Anat Record 101 373–405
89 ALLEN J G 1952 The pathogenesis of irradiation hemorrhage Trans 5th Conf Blood Coagulation J E Flynn Josiah Macy Jr Foundation New York N Y
90 SWISHER S N 1954 Personal communication
91 *SHECHMEISTER I L V P BOND & M N SWIFT 1952 The susceptibility of ir*radiated mice to infection as a function of postradiation time J Immunol 68 87–95
92 HAMMOND C W M COLLING D B COOPER & C P MILLER 1954 Studies on susceptibility to infection following ionizing radiation II Its estimation by oral inoculation at different time post irradiation J Exptl Med 99 411–418

93 SHECHMEISTER I L & M FISHMAN To be published
94 FISHMAN M & I L SHECHMEISTER To be published
95 VALENTINE W N M L PEARCE & J S LAWRENCE 1952 Studies on the radiosensitivity of bone marrow II The effect of large repeated whole body irradiation exposure on hematopoiesis Blood 7 14-19
96 STRANGEWAYS T S P & F L HOPWOOD 1926 Effect of X rays upon mitotic cell division. Proc Roy Soc London 100(B) 283-293
97 CARLSON J G 1942 Immediate effects of 31 r of X rays on the different stages of mitosis in neuroblasts of chortophaga J Morphol. 71 449-462
98 BRUES A M & L REITZ 1951 Effects of external and internal radiation on cell division Ann N Y Acad Sci 51 1497-1508
99 SPARROW A H 1951 Radiation sensitivity of cells during mitotic and meiotic cycles with emphasis on possible cytochemical changes Ann N Y Acad Sci 51 1508-1541
100 WIDNER W R J B STORER & C C LUSHBAUGH 1951 The use of X ray and nitrogen mustard to determine the mitotic and intermitotic times in normal and malignant rat tissues Cancer Research 11 877-884
101 SCHLANG H A 1952 Schwartzman phenomenon III Modifications of nitrogen mustard suppression Proc Soc Exptl Biol. Med 81 274-277
102 SCHLANG H A E P CRONKITE & G BRECHER 1953 Some experimental approaches to the therapy of whole body irradiation Lecture and review series No 53-1 Naval Med Research Inst Bethesda Md
103 SMITH F W W SMITH H L ANDREWS M M GREVAN & I ALDERMAN Effect of parenteral injection of particulate matter on survival in X irradiated animals Unpublished data
104 CRONKITE E P Unpublished data
105 FARR R S Personal communication of unpublished data
106 STRUMIA M M Personal communication of unpublished data
107 HENKEL, D T P B MEFFERD JR & J B LOEFFER 1953 Effect of a protein free bacterial pyrogen on leukocytic counts in irradiated mice Texas Repts Biol Med 11 495-501
108 STORER J B P SAUNDERS & C C LUSHBAUGH 1952 Effect of leukocyte promoting factor (LPF) on survival of X irradiated mice Proc Soc Exptl Biol Med 81 541-542
109 HENSHAW P S 1938 Studies on the effect of roentgen rays on the time of the first cleavage in some marine invertebrate eggs I Recovery from roentgen ray effects in Arbacia eggs Am J Roentgenol Radium Therapy 27 890 898
110 HOLLAENDER A W K BAKER & E H ANDERSON 1951 Effect of oxygen tension and certain chemicals on the X ray sensitivity of mutation production and survival. Cold Spring Harbor Symposia Quant. Biol 16 315-325
111 MCKINNEY G R S P MARTIN R W RUNDLES & R GREEN 1953 Respiratory and glycolytic activity of human leukocytes *in vitro* J Applied Physiol 5 335
112 BRECHER G K M WILBUR & E P CRONKITE 1953 Transfusion of separated leukocytes into irradiated dogs with aplastic marrows Proc Soc Exptl. Biol Med 84 54-56
113 BRECHER G & E P CRONKITE 1953 The effects of platelet transfusions in dogs made pancytopenic by X radiation N Y State J Med 53 544-547
114 CRONKITE E P G BRECHER & K M WILBUR 1954 Development and use of a canine blood donor colony for experimental purposes I Leukocyte and platelet transfusions in irradiation aplasia of the dog bone marrow Military Surgeon 114 359-365

Part III Factors Influencing Numbers, Distribution, and Fate of Leukocytes

DAILY RHYTHMS IN NUMBERS OF CIRCULATING EOSINOPHILS AND SOME RELATED PHENOMENA

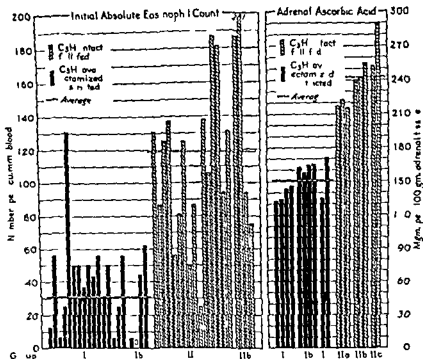
By Maurice B Visscher and Franz Halberg
Department of Physiology University of Minnesota Minneapolis Minn

Our interest in the problems suggested by the title of this report arose by an accident of observation We were investigating the functional activity of the adrenal cortex in mice in which hyperplasia and metaplasia had been induced by gonadectomy Earlier studies (King Casas and Visscher[1]) had shown that dietary calorie restriction in such female mice of the Z (C_3H) strain prevented the characteristic estrogen secretion leading to continuous subestrus, but did not prevent the morphologic changes in the adrenal gland It seemed of importance to determine whether the normal hormones of the adrenal cortex were or were not being secreted in increased amount in the mouse with castration induced adrenal change either in the full fed or the calorie restricted state Therefore, studies were made of the numbers of circulating eosinophils and of the adrenal gland ascorbic acid concentration as possible indices of adrenal activity

A first experiment appeared to indicate an increased adrenal cortical activity in the calorie restricted ovariectomized mice, as compared with the intact full fed mice FIGURE 1 shows the data of this experiment On the average the eosinophil counts were lower in the operated than in the intact mice A similar difference was then seen in adrenal ascorbic acid concentration Moreover, the adrenals of the ovariectomized restricted group were heavier (as was to be expected) No overlap was seen between the weight ranges of the two groups studied, but repetitions of the eosinophil counts on corresponding groups of mice failed to corroborate the finding of the first experiment It became obvious that an unknown variable was playing a determining role in the results at least as far as eosinophil counts were concerned A review of the protocols revealed that the successive experiments were begun at different times of day

It was recognized later that under the conditions of these experiments the inconsistency of the results may have been brought about by a lag in phase between the eosinophil rhythms of the two groups In view of the results presented below it appears fair to assume that the cycle of the full fed mice was synchronized with the lighting regimen and that of the restricted mice with the feeding regimen

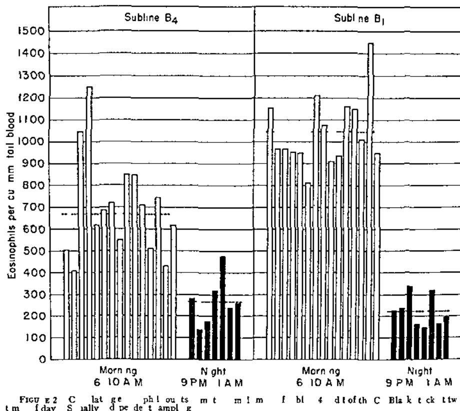
A series of studies was conducted in order to test the possibility that factors related to time of day are critical in determining the level of circulating eosinophils in mice The results[2] of such a test on mice of two sublines of the C_{57} Black strain of mice are shown in FIGURE 2 The existence of a daily cycle in eosinophil levels has also been noted on other strains of mice (TABLE 1) on rats, on dogs and on man[3] but not on sheep[4] The large daily variations



Fi ure 1 Pre t ly u p bl h d bserv t s lat g os oph l l adren l sc b c a d con n- t t n xpe m t l and o t l groups fm The low rm n noph l u t o tom zed t t d C₃H m a d th low adre l sc b c d n t t tat t lly s gn fi t b t th d ff re re n t sc b bl t the n ble u de t ly e th pla m t l th d rnal y le w ot c t li d

which occur in the level of circulating eosinophils are fairly regular as to timing under standardized experimental circumstances and it became immediately apparent that no study involving this variable could ignore these variations

Since the eosinophil rhythm appeared to have a 24-hour cycle length it became obvious that the physical environmental factors which exhibit daily cycles might contribute to the establishment maintenance and/or timing of the rhythm Temperature variations were ruled out because the mice were housed with thermostatic control Lighting cycles obviously might be involved and were therefore tested as determinants TABLE 2 presents the data from a set of experiments in which mice were exposed to a reversal of lighting schedules that is a shift from daytime light to night time (6 P M to 6 A M) light exposure[5] It will be noted that after four days of reversal of lighting the previously statistically significant lower eosinophil level at night was not seen and that at eight days a statistically significant phase reversal was established It is further apparent from this table that on *ad libitum* feeding the *factor of the time at which food was available was not critical for the eosinophil* cycle phase relationship In these and other studies mice were singly caged and kept at 78 ± 1 F and fed on Purina Fox Chow Moreover we have employed serially independent sampling for day night difference studies Thus



FIGU E 2 C lat ge ph l ou ts m t m l m f bl 4 d l of th C Bla k t ck t tw t m f day S ially d pe de t ampl g

out of a group of mice one half selected at random would be subjected to blood study at night and the other half would be studied about 12 hours later It has been shown that venipuncture in the mouse tail brings about a significant alteration of eosinophil behavior the effect of which may be seen after a period of several days [6] thus making necessary more elaborate controls of the effect of the sampling procedure itself when serially dependent sampling is employed

The effect of exposure to continuous darkness was tested in order to ascertain whether the rhythm would persist without daily reinforcement by a lighting cycle The fact that reversal in the latter did not immediately result in phase reversal of the eosinophil rhythm suggested the probable result of the test As TABLE 3 shows the cycle persists for at least 13 days of continuous darkness in *ad libitum* fed mice

Although as noted in TABLE 2 the time of feeding is not critical when food is *freely* available for 12 hours or more per day it can be seen from TABLE 4 that when food is limited to about two thirds of the calories ingested by mice of the same age when allowed free access to food there is a complete reversal of cycle which so to speak turns night into day for the mouse when a limited amount of food is available only by day This reversal has not been fully established at 14 days after establishment of the feeding regimen thus indicating a longer

TABLE 1

EOSINOPHIL RHYTHM IN MICE OF BOTH SEXES OF VARIOUS AGES AND STOCKS WITH VARIOUS CHARACTERISTIC EOSINOPHIL LEVELS

Stock	Age (months)	Time of sampling	No. of mice	Mean No. of eos. and S.E.	Difference and S.E.	t	P
Males							
AxZl F	1½	Day Night	19 19	182 ± 15 40 ± 5	141 ± 16	9.04	<0.001
B	6	Day Night	14 14	1052 ± 43 228 ± 16	824 ± 46	17.83	<0.001
B	25	Day Night	14 14	736 ± 97 301 ± 49	435 ± 113	3.84	<0.001
B_4	6	Day Night	15 13	700 ± 60 274 ± 26	426 ± 68	6.25	<0.001
B_4	25	Day Night	10 9	678 ± 61 458 ± 51	221 ± 85	2.60	0.019
D	6	Day Night	14 13	297 ± 47 95 ± 19	201 ± 47	4.31	<0.001
ZD_8	6	Day Night	14 9	130 ± 14 41 ± 8	89 ± 19	4.65	<0.001
C H	6	Day Night	5 5	165 ± 12 15 ± 7	150 ± 14	11.06	<0.001
Ce (I)	18	Day Night	8 8	679 ± 73 287 ± 47	392 ± 93	4.22	<0.001
Ce (II)	21	Day Night	17 17	536 ± 35 409 ± 28	126 ± 47	2.71	0.010
Ax	6	Day Night	14 13	169 ± 15 43 ± 7	124 ± 16	7.56	<0.001
A	6	Day Night	14 13	166 ± 21 90 ± 14	76 ± 26	2.97	0.007
Zl	6	Day Night	14 13	198 ± 19 31 ± 4	167 ± 20	8.46	<0.001
Z	6	Day Night	14 11	179 ± 17 60 ± 13	120 ± 23	5.26	<0.001
Females							
ZBC (I)	1½	Day Night	10 10	83 ± 17 31 ± 4	52 ± 18	2.94	0.010
ZBC (II)	1½	Day Night	10 10	109 ± 17 43 ± 6	67 ± 18	3.68	0.007
ZBC (III)	4	Day Night	15 11	162 ± 20 79 ± 14	83 ± 26	3.27	0.004

TABLE 1—*Concluded*

Stock	Age (months)	Time of sampling	No. of mice	Mean No. of eosinophils and S.E.	Difference and S.E.	t	P_t
Females							
ZBC (IV)	4	Day Night	8 9	77 ± 12 36 ± 4	41 ± 12	3.50	0.003
C_3H	5	Day Night	5 5	104 ± 21 28 ± 14	76 ± 25	2.97	0.018
$AxZbF_1$	16	Day Night	10 11	168 ± 38 21 ± 4	146 ± 37	3.97	0.001
Ce	18	Day Night	10 10	609 ± 43 327 ± 40	282 ± 62	4.52	<0.001
B_4	21	Day Night	5 5	717 ± 99 300 ± 49	417 ± 123	3.38	0.009

TABLE 2

REVERSAL OF EOSINOPHIL RHYTHM BY REVERSAL IN ILLUMINATION IN MALE CBC MICE TWO MONTHS OF AGE ON VARIOUS FEEDING REGIMENS

Days after reversal of illumination	Food available	Time of sampling	No. of mice	Mean eosinophil ± S.E.	Difference ± S.E.	t	P_t
0 0	Ad lib	D N	9 10	245 ± 39 65 ± 10	+180 ± 39	4.66	<0.001
2 3	Ad lib	D N	10 10	211 ± 30 99 ± 12	+111 ± 32	3.46	0.003
4 5	Ad lib	D N	10 10	125 ± 30 177 ± 12	−52 ± 32	1.61	0.116
8 9	Ad lib	D N	10 10	123 ± 19 337 ± 45	−214 ± 49	4.40	<0.001
8 9	Ad lib during day (dark)	D N	10 10	80 ± 21 211 ± 29	−131 ± 36	3.68	0.002
8 9	Ad lib during night (light)	D N	10 10	78 ± 19 287 ± 24	−209 ± 30	6.93	<0.001
17 18	Ad lib during day (dark)	D N	9 9	87 ± 18 250 ± 51	−163 ± 54	3.03	0.008
17 18	Ad lib during night (light)	D N	10 10	100 ± 19 358 ± 47	−257 ± 51	5.09	<0.001
0 0	Ad lib	D N	9 9	373 ± 58 84 ± 14	+290 ± 60	4.85	<0.001

TABLE 3
EOSINOPHIL RHYTHM IN AD LIBITUM FED MALE ZBC MICE TWO MONTHS OF AGE KEPT IN CONTINUOUS DARKNESS

Lighting	Days in dark	Time of sampling	No. of mice	Mean No. of eosinophils ± S E	Difference ± S E	t	P
Continuous darkness	7 8	D N	8 8	163 ± 27 37 ± 14	126 ± 30	4 21	<0 001
Alternating light dark	0	D N	7 8	183 ± 41 39 ± 10	144 ± 44	3 24	0 006
Continuous darkness	13	D N	22 21	210 ± 23 81 ± 16	129 ± 28	4 60	<0 001
Alternating light dark	0	D N	22 23	256 ± 32 54 ± 9	202 ± 33	6 12	<0 001

Light on at 06 00 h and off at 18 00 hours

TABLE 4
EFFECT OF TIME OF FEEDING UPON EOSINOPHIL RHYTHM IN FEMALE C_3H MICE RESTRICTED IN CALORIC INTAKE

Diet	Time of feeding	Duration of feeding regimen	Time of sampling	No. of mice	Mean No. eosinophils ± S E	Difference ± S E	t	P
Restricted	09 30	45 days	D N	18 7	42 ± 9 113 ± 31	72 ± 24	3 03	0 006
Restricted	18 30	102 days	D N	15 17	186 ± 18 6 ± 1	180 ± 17	10 69	<0 001
Restricted	09 30	14 days	D N	12 14	62 ± 9 45 ± 7	17 ± 11	1 53	0 147
Restricted	09 30	44 days	D N	4 5	7 ± 1 82 ± 9	75 ± 10	7 21	0 001
Purina Fox Chow	Ad lib	Since weaning	D N	14 12	114 ± 27 24 ± 4	89 ± 30	3 02	<0 006

From 08 30 hours to 09 30 hours

lag period than was seen with lighting changes Mice given food *ad libitum* throughout the 24 hour period feed much by night and are more active by night under the conditions prevailing in the laboratory The calorie restricted mouse fed during the day is very much more active than are *ad libitum* fed mice during the day It appears that in some way, the eosinophil cycle is related to activity rhythms[3] Severe calorie restriction makes the period of feeding an adequate timer of some fundamental rhythm in the mouse Since this is not the case in full fed mice it is unlikely that alimentation and absorption *per se* are responsible More likely a more subtle effect of nutrient want is involved perhaps the effect of hunger upon mechanisms controlling activity levels

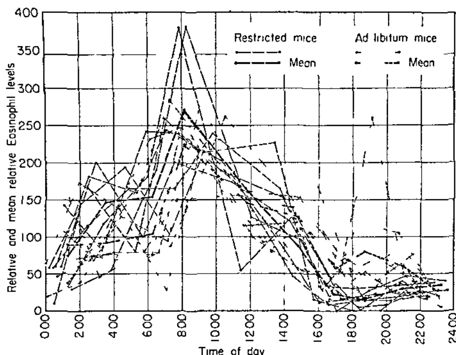
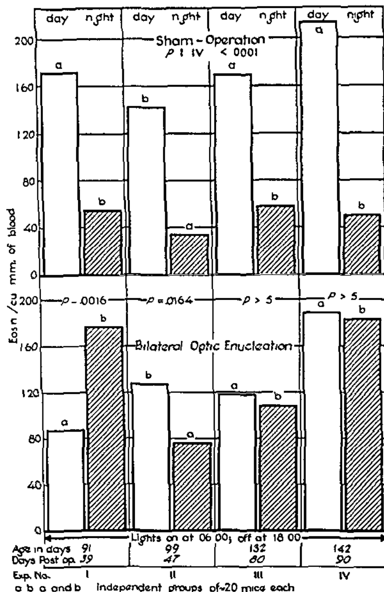


FIGURE 3 Pe s st f ph l hythm n m *d l b t m* f d d se ely t ct d ı d tary lor f r se l m th S r ally d pend t sampli g

The data presented till now were derived from serially independent studies on mice sampled either by day or at night. It may properly be asked whether those particular times are optimal for diurnal cycle studies and whether the cycle can be observed in individual mice studied at intervals of several days at various times of day Such serially dependent observations as shown in FIGURE 3 were made in *ad libitum* fed and calorie restricted animals fed in the evening (18 30)[7] The mice were on a regimen providing for light by day and darkness by night Despite some random variability it will be seen that there is a very regular daily high at about 8 A M and a low during a period of several hours before midnight Furthermore it may be noted that the amplitude of the cycle is not less (in fact it is somewhat greater) in the calorie restricted than in the full fed mice It is noted that this degree of caloric restriction abolished the estrus cycle of comparable groups of mice in studies by Puh Lee and Visscher[8]

Since lighting cycle reversal produced a sharp reversal in eosinophil rhythm it was decided to test the role of the eyes in the phenomenon Bilateral optic enucleation was performed and counts were made on blood from operated and from 'control' mice using serially independent sampling The results are seen in FIGURE 4 In the sham operated animals a normal cycle persisted for the duration of the study After blinding an inverted cycle was seen at the 39th postoperative day reversing again by the 47th At 80 and 90 days after



Γ 4 M ph I I els t tw t m f d y t l blood f m bl d d d f m ham-ope t l l CBC m d g th th m th f llow g th pe t 5 lly d pe d t sampl ng N t th t ded m (below) d t h b t th gul r beh f h m-ope t d m (bo) (1) th d ti f y ght h g e sed t 3) d y ft bl d g ( ) th d y ght d ff m t t gn fi t tat t lly t 80 d t 90 d y ft bl d g

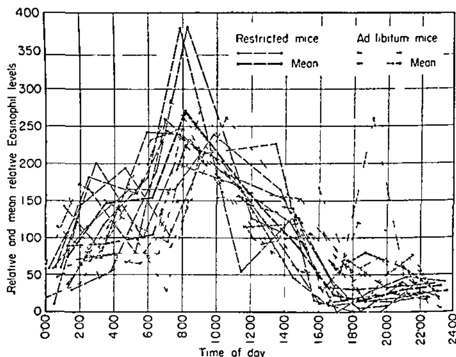


FIGURE 3 Pe st ce f eos ph l hythm n m ce *ad l b t m* f d d se er ly rest i t d d t ry calori for sev l m th Se lly d pe d t sampl g

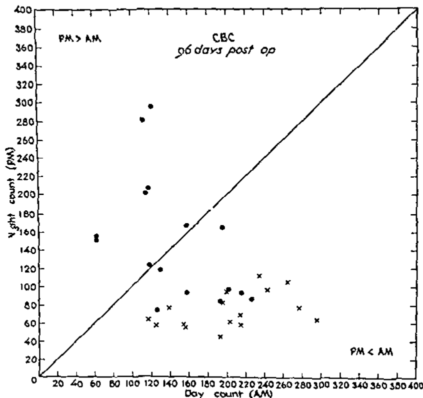
The data presented till now were derived from serially independent studies on mice sampled either by day or at night. It may properly be asked whether those particular times are optimal for diurnal cycle studies and whether the cycle can be observed in individual mice studied at intervals of several days at various times of day Such serially dependent observations, as shown in FIGURE 3 were made in *ad libitum* fed and calorie restricted animals fed in the evening (18 30)[7] The mice were on a regimen providing for light by day and darkness by night Despite some random variability it will be seen that there is a very regular daily high at about 8 A M and a low during a period of several hours before midnight Furthermore it may be noted that the amplitude of the cycle is not less (in fact it is somewhat greater) in the calorie restricted than in the full fed mice It is noted that this degree of caloric restriction abolished the estrus cycle of comparable groups of mice in studies by Puh Lee and Visscher[8]

Since lighting cycle reversal produced a sharp reversal in eosinophil rhythm, it was decided to test the role of the eyes in the phenomenon Bilateral optic enucleation was performed and counts were made on blood from operated and from "control mice using serially independent sampling The results are seen in FIGURE 4 In the sham-operated animals a normal cycle persisted for the duration of the study After blinding an inverted cycle was seen at the 39th postoperative day reversing again by the 47th At 80 and 90 days after

that the act of observation would vitiate or at least alter subsequent observations on the same animals

Figures 5a and b present the records of mean rectal temperatures in sham operated and optically enucleated mice for 90 postoperative days It will be noted that the blinded mice show a shortening of cycle length so that there is a phase reversal at about 15 days with a return to the original phase relationship at 33 days Furthermore by 48 days there is a decrease in amplitude of cycle in the blinded mice treated as a group and with the mean values plotted However, when data from individual mice are studied it is apparent that cycles of approximately normal amplitude existed but that they were so randomized as to timing that the mean showed no significant cycle for several days

A more refined mathematical study using the method of periodogram analysis[9] on the data obtained during the first month after operation shows that the



F 6 Eo ph l ou t t two t m f d y ta l l lood f om bl d d d f om h m perat d m l CBC m e 96 day f the perat Sen lly d pe d t samp) g t random zatio f probably cy f ph l beh d d l bl nd d m By t t th ght t of th h m pe t d m re co te tly low th the day ts F G e 6 (bov) CBC m F e 6 (t pag) DBA m

The Effect of Bilateral Optic Enucleation Upon the 24-Hour Rhythm in Rectal Temperature of Male CBC Mice

FIGURE 5

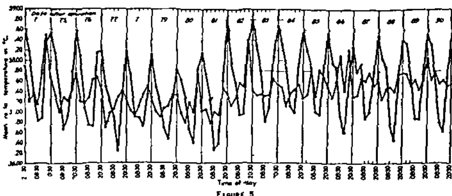


FIGURE 5

F GUR 5 Th ff t f b lateral pt c l at n pon m ct l t mpe t f mal CBC m Fi ur 5 Obser at o s d g tw m ths f llow g per t n F GUR 5B Observ t d r g the th d m th follow ng pe ati

blinding statistically significant day night differences in mean counts were not seen

These results appear to be confusing at first glance but they are much clarified by consideration of some concomitant observations on body tempera ture cycles The latter were performed because rectal temperature measure ments by means of a thermistor seemed to be less traumatic procedures than venipunctures and it was hoped that they could be carried out with less danger

become dominant, but an intrinsic rhythm of cycle length somewhat less than the solar day seems to be a persistent phenomenon Whether this rhythm which survives optic enucleation is inherent in the sense of being inborn or is conditioned by environmental stimuli of modalities other than light is unknown The fact that congenitally blind mice may show a solar day rhythm

TABLE 5

EFFECT OF BILATERAL ADRENALECTOMY AND OF BILATERAL ADRENALECTOMY PLUS GONADECTOMY UPON EOSINOPHIL RHYTHM IN MALE CBC MICE TWO MONTHS OF AGE

Day It pe tion	Pe od f sampl g	No	M N os o ph ls ± S E	Diff re ± S E	t	P
			Bilateral adrenalectomy			
2	D N	17 17	177 ± 36 166 ± 19	11 ± 42	0 26	>0 500
4	D N	16 15	315 ± 50 291 ± 34	24 ± 64	0 38	>0 500
9	D N	11 11	572 ± 83 429 ± 90	144 ± 128	1 12	0 284
			Bilateral adrenalectomy plus bilateral gonadectomy			
2	D N	18 16	128 ± 22 176 ± 28	47 ± 37	1 29	0 194
4	D N	15 14	339 ± 47 329 ± 60	10 ± 79	0 13	>0 500
9	D N	7 5	491 ± 87 452 ± 56	39 ± 124	0 31	>0 500
			Sham adrenalectomy			
2	D N	18 17	261 ± 29 108 ± 19	152 ± 36	4 18	<0 001
4	D N	18 17	277 ± 30 106 ± 15	171 ± 35	4 84	<0 001
9	D N	12 11	230 ± 34 81 ± 10	149 ± 39	3 85	0 001
			No operation			
2	D N	18 17	296 ± 40 124 ± 17	172 ± 46	3 76	<0 001
4	D N	17 17	256 ± 34 107 ± 11	149 ± 37	4 00	<0 001
9	D N	12 11	187 ± 22 53 ± 7	129 ± 25	5 10	<0 001

Alt ta t f pe me t

blinded mice have cycle lengths of about 23⅓ hours as compared with the cycle lengths of roughly 24 hours in the controls At three months there appears to be more randomness because some of the blinded mice still have cycle lengths that average less than the solar day, while other blinded mice again exhibit 24 hour cycles

These temperature observations have obvious bearings on the eosinophil data mentioned previously It is apparent that a reversal in phase may occur in blinded mice simply because of a shortening (or lengthening) of cycle length Further an apparent obliteration of cycle, as judged from mean values may be a complete artifact due to randomization in animals in which the usually *dominant synchronizer is lost* (FIGURES 6A *and* B) *Lighting effects through* the eyes appear to be the dominant normal synchronizer of the 24 hour cycle in mice In the absence of this mechanism other environmental factors may

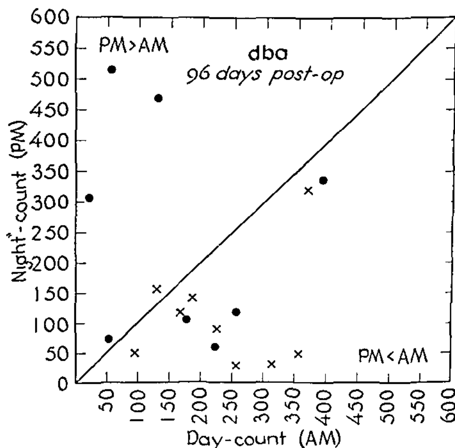
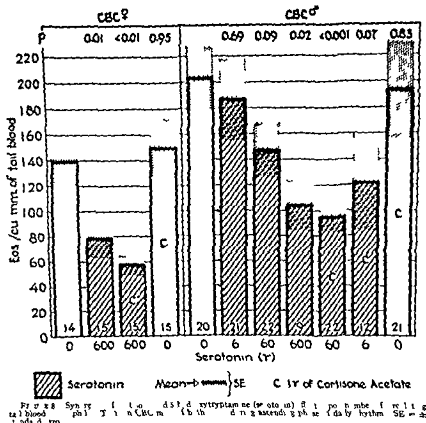


FIGURE 6B

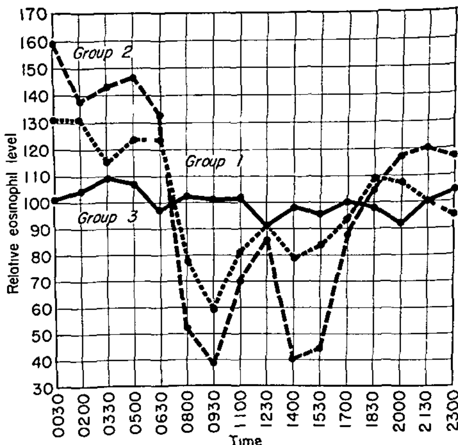


Fɪɢᴜʀᴇ 8 Syn rg f t o d 5 ? d xytryptam ne (se oto in) fl t po n mbe f re l t g ta l blood ph l T 1 n CBC m f b th d n g ascendi g ph se f da ly hythm SE = ± t nda d rro

ation in mean eosinophil counts as a group phenomenon.[3] Furthermore since compounds F and F produce eosinopenia it is easy to suggest that their increased output at certain times of day produces the characteristic morning eosinopenia in man and the corresponding night low in mice. However the problem may not be so simple because other factors may easily be of equal importance. Recent observations by Halberg[10] show that serotonin sharply potentiates the eosinopenic action of cortisone. Fɪɢᴜʀᴇ 8 demonstrates this effect clearly. Since serotonin is a substance known[11] to be in relatively high concentration in nervous tissue the possibility must not be ignored that it may be liberated under certain circumstances and exert a hormonal action. This observation also points up the fact that biological assays are obviously unreliable unless performed with highly purified materials if potentiating substances for the material to be assayed are present. Thus one cannot with any assurance use for example the eosinopenic action of blood as a measure of its 11–17 oxysteroid content unless the presence of potentiators is first excluded

and that it is inverted by reversal of lighting if "seeing" mice are in the same room and not otherwise, indicates a dependence in such blind mice upon sense organs other than the eye It does not help to decide definitely as to whether the basic cyclic phenomenon is inherited or acquired Surely, the capacity for the cycles is independent of vision and is inherited, but it seems most likely that the development of a basic cycle length of approximately a solar day is conditioned by the repetition, at such intervals of sensory inflow peaks in the developing animal

The precise mediating mechanisms for cyclic variations in level of blood eosinophils are not known with certainty Removal of the adrenal glands in mice and untreated cortical insufficiency in man appear to abolish the characteristic cycle TABLE 5 presents data on mice showing no significant day night difference after bilateral adrenalectomy, with or without gonadectomy FIGURE 7 shows that, in human subjects with severe untreated adrenal cortical insufficiency in contrast to normal subjects there is no significant cyclic vari



FIGU 7 V t d r g the 24 h pe od m l t rb l l l m G oup 2 (b k l) Unlim t d a t ty G p 1 (d tt d l) Lim ted t ty G p 3 (sol d l) L m t d t ty ad nal suffi cy

20 Mickelsen O E V O Miller & A Keys To be published
21 Speck C 1887 Arch exptl Path Pharmakol 16 81
22 Leathes J B 1906 J Physiol 35 175
23 Cathcart E P F I Kennaway & J B Leathes 1908 Quart J Med 1 416
24 Elmadjian F & G Pincus 1946 J Clin Endocrinol 6 287
25 von Euler U S & V C Holmquist 1934 Arch ges Physiol 234 210
26 Lehmann G & H F Michaelis 1943 Arbeitsphysiol 12 305
27 Döring G K & I Schaefers 1950 Arch ges Physiol 252 537
28 Rosenbaum J D B C Ferguson R K Davis & F C Rossmeisl 1952 J Clin Invest 31 507 F Gerritzen 1940 Acta Med Scand Suppl 108 121
29 Bullough W S 1952 Biol Revs Cambridge Phil Soc 27 133
30 Halberg F H A Zander M W Houglum & H R Muhlemann 1954 Am J Physiol 177 361
31 Barnum C P & F Halberg 1953 Metabolism Clin & Exptl 2 271
32 Halberg F C P Barnum & H Vermund 1953 J Clin Endocrinol Metab 13 871
33 Calhoun J B 1944 J Tenn Acad Sci 19 252 179 1945 J Tenn Acad Sci 20 228 291 1946 J Tenn Acad Sci 21 208 281
34 Welsh J H 1938 Quart Rev Biol 13 123
35 Jores A 1938 Deut med Wochschr 64 737 989
36 Park O 1940 Ecol Monographs 10 485
37 Rud F 1947 The Eosinophil Count in Health and Mental Disease a Biometrical Study Johan Grundt Tanum Forlag Oslo Norway
38 Brown F A Jr & H M Webb 1948 Physiol Zoöl 21 371
39 Kleitman N 1949 Physiol Revs 29 1
40 Studium Generale Zeitschrift f d Einheit der Wissenschaften im Zusammenhang ihrer Begriffsbildungen und Forschungsmethoden 1949 2 67 135
41 Menzel W 1952 Z Altersforschung 6 26 104

but biochemical measurements of hydroxycorticoids in blood and in urine have revealed daily rhythms,[12 15] corresponding to earlier observations on the renal excretions of 17 ketosteroids,[16 0] neutral reducing lipids[19] and uric acid[21 3] which are less by night than by day Twenty four hour periodicity has also been noted for the levels of circulating lymphocytes[4] and for several indices of medullary adrenal function[25 7] Further work will perhaps elucidate the exact role of neurohumors acting against a background of corticoid activity in producing the evident cycles in eosinophil levels, body temperature, and other body functions and characteristics[3]

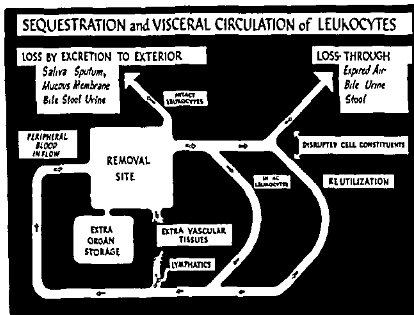
The daily rhythms in circulating eosinophil level and in body temperature are not the only, nor necessarily the more important cycles of solar day length among physiologic parameters Diurnal cycles in salt excretion,[28] in mitotic activity,[29 30] and even in the relative specific activity of phospholipid phosphorus of defined cell fractions[31 3] are examples of other such rhythms Available evidence points to humoral mechanisms for their mediation and for their establishment as a result of repetitive exposure of the individual to regular changes in intensity of some environmental stimulus or stimuli For additional data and discussions related to these problems, the interested reader may be referred to other publications[3 33-41]

Acknowledgment

The authors are indebted to the editors of *The American Journal of Physiology Endocrinology The Journal Lancet* and the *Proceedings of the Society for Experimental Biology and Medicine* for permission to include herein some materials which have been published earlier

References

1 Casas C B J T King & M B Visscher 1949 Am J Physiol 157 193
2 Halberg F 1951 Proc 2nd Clin ACTH Conf 1 84 J R Mote Ed Blakiston New York N Y F Halberg & M B Visscher 1950 Proc Soc Exptl Biol Med 75 846 H Panzenhagen & R Speirs 1953 Blood 8 536 C Louch R K Meyer & J T Emlen 1953 Proc Soc Exptl Biol Med 82 668
3 Halberg F 1953 J Lancet 73 20
4 Zarrow M X M E Denison B Rosenberg D E Mann Jr & G M Neher 1952 Am J Physiol 171 636
5 Halberg F M B Visscher & J J Bittner 1953 Am J Physiol 174 109
6 Halberg F 1953 Proc Soc Exptl Biol Med 82 160
7 Halberg F & M B Visscher 1952 Endocrinology 51 329
8 Puh Lee Y C & M B Visscher 1952 Unpublished data
9 Whittaker E T & G Robinson 1924 The Calculus of Observations 343–362 Blackie & Son London England
10 Halberg F 1954 Am J Physiol In press
11 Twarog B M & I H Page 1953 Am J Physiol 175 157
12 Appel W & K J Hansen 1952 Deut Arch klin Med 199 530
13 Doe R P E B Flink & M G Flint 1954 J Clin Endocrinol Metab 14 774
14 Tyler F H C Migeon A A Florentin & L T Samuels 1954 J Clin Endocrinol Metab 14 774
15 Sandberg A A D H Nelson E M Glenn F H Tyler & L T Samuels 1953 J Clin Endocrinol Metab 13 1445
16 Pincus G 1943 J Clin Endocrinol Metab 3 195
17 Wooster H 1943 J Clin Endocrinol Metab 3 483
18 Forbes A P E C Donaldson E C Reifenstein Jr & F Albright 1947 J Clin Endocrinol Metab 7 264
19 Pincus G L P Romanoff & J Carlo 1948 J Clin Endocrinol Metab 8 221



F[illegible]URE 1 S[illegible]h[illegible]m[illegible]t[illegible] represe[illegible]t[illegible]tio[illegible] of seq[illegible] [illegible] and [illegible] sce[illegible] l c[illegible]rculatio[illegible] [illegible]f le[illegible]kocyte[illegible] i[illegible] m[illegible] It sh[illegible]uld be [illegible] t[illegible] p[illegible] t[illegible]d that co[illegible] derable [illegible] w[illegible]ll oc[illegible] depe[illegible]ding [illegible]po[illegible] the type [illegible] d[illegible] m[illegible] t[illegible] [illegible]ty [illegible]f th[illegible] leuk[illegible] cyte [illegible] d th[illegible] [illegible] t[illegible] i[illegible] [illegible]ol[illegible] d

this extremely dynamic situation it is entirely possible that, at times the balance may be in favor of production and at other times in favor of removal Data strongly suggest that impairment of removal from the peripheral blood or destruction of the tissues in the leukemias may occur more often than has hitherto been seriously considered[2] On the basis of impaired removal it was predicted that the intravascular life span of the predominant leukocyte type in the leukemias and perhaps the extravascular phase also, would be prolonged over that of the usually accepted normal figures This prediction seems to be partly verified by the findings of a prolonged span of life of the lymphocyte in chronic lymphocytic leukemia

To complicate the situation further the sites which are capable of withdrawing leukocytes from the circulation are many are situated in widely separated locations and possess divergent capabilities in dealing with leukocytes To date the lung liver spleen gastrointestinal tract bone marrow striated muscle and kidney have been implicated as leukocyte removal sites (FIGURE 1)

Lung

The pulmonary circulation was found capable of rapidly removing and delivering vast quantities of leukocytes from and into the circulating blood as well as storing and destroying leukocytes[2, 3] The accessibility of the lung for

THE SEQUESTRATION AND VISCERAL CIRCULATION OF LEUKOCYTES IN MAN

By Howard R Bierman Keith H Kelly, and Fauno L Cordes
Division of Research City of Hope Medical Center Duarte Calif

The rapidly changing conditions of hematological equilibrium that have been encountered require a more dynamic approach to this problem than was anticipated during the initial phases of these investigations The definition of sequestration of leukocytes therefore has been expanded to encompass the dynamic concept of hematologic equilibrium and to include the process of withdrawal of leukocytes from the peripheral circulation, selectively or collectively, the storage transfer, and destruction of the intact leukocyte in this site, the ultimate fate and destiny of the remaining intact leukocytes, and the products of disruption of the destroyed leukocytes (FIGURE 1)

The life span of the leukocytes may be arbitrarily divided into three phases (FIGURE 2) They are (1) the hematopoietic phase—that period from the initiation of the development of the leukocyte from its basic primitive cell to the point at which it is delivered into the peripheral circulation, (2) the intravascular phase—the duration that the leukocyte spends in the peripheral circulation, and (3) the extravascular phase—that period of time the leukocyte spends out of the peripheral circulation in viscera or in the tissues proper Although these three phases appear distinct the boundaries are probably not intact and invite a free flow of leukocytes back and forth with surprising ease and rapidity

Each type of leukocyte under discussion—be it the neutrophil, eosinophil basophil monocyte or lymphocyte—probably has a different life span Furthermore it is likely that the life span of the immature forms of these specific types if they make their way into the peripheral circulating blood may also differ from those of the mature form of the same type The life span of a leukocyte therefore does not exist *per se* but would be a mean value of all the cells The free access of leukocytes to the various hematological compartments in all probability influences the life span of any single cell type material and renders a mean life span of all cells of lesser value than is generally considered

The extravascular store of leukocytes is extremely large in number One can liken the peripheral circulating blood to the tip of a floating iceberg, in that one merely sees a small part of the entire hematological picture in the peripheral circulation while the major functions are in the hemapoietic and extravascular regions which are beneath the surface That this extreme variability and dynamic state is present in the normal individual but is under control and is unchecked in the abnormal leukemic state may account for the wide variations which are observed in the peripheral blood and tissues

It has been suggested previously that the normal hematological state is a reflection of the equilibrium between production and removal and that the leukemias may exhibit an unbalance between production and removal[1] With

levels obtained from the inflow and outflow tracts of the lung were observed to rise The arterial leukocyte rise preceded and exceeded that of the venous samples and consequently the leukocytes were presumed to come from the lungs

When the normal expiration was exaggerated by the Valsalva maneuver there was a prompt and often profound drop in leukocyte number in the arterial blood which persisted throughout the forced expiration A slow rise and then fall was observed in the venous blood samples if the expiration was prolonged enough The pulmonary circulation therefore is capable of withdrawing vast numbers of cells at a most rapid rate under these circumstances and yet upon release of the forced expiration these large numbers of cells are apparently not returned to the circulation in a similar fashion Under these conditions the rate of removal of leukocytes from the blood by the lung temporarily exceeds the rate of delivery of leukocytes by the rest of the body The net result is a loss of leukocytes from the circulation With the release of the forced expiration the arterial and venous counts return to the control level with little if any overshoot The major portion of the leukocytes trapped in the pulmonary circulation during the Valsalva maneuver therefore remain unaccounted for

The lungs of nonleukemic patients are most efficient removers of leukocytes from the circulation as demonstrated by the infusion of large numbers of leukocytes obtained from leukemic donors The results were essentially the same whether the leukocytes were given rapidly in a single short infusion over a period of 2 to 10 minutes or administered by a prolonged infusion of tremendous numbers of leukocytes by arterial cross-transfusion over periods of 6 to 35 hours The normal pulmonary circulation proved capable of removing practically all such infused leukocytes In contrast the similar infusion of leukocytes into leukemic recipients was usually but not always associated with the passage of more leukocytes through the lung than were found with the nonleukemic recipients Similar findings occurred occasionally in the nonleukemic recipient and in subleukemic recipients practically no infused cells passed through the lungs Upon cessation of the leukocyte infusion there was no compensatory rise in the arterial number of leukocytes to account for those leukocytes trapped in the pulmonary circulation On the contrary the normal recipient in such cross-transfusions frequently exhibited a further decrease in the leukocyte number some hours after the cross-transfusion was terminated The prior administration of heparin in doses up to 6 mgm per kilo body weight apparently made it possible for approximately 10 to 30 per cent of the anticipated number of leukocytes to enter the arterial circulation following the transfusion of large numbers of leukocytes With the return of the clotting power toward normal there was a prompt withdrawal of these leukocytes from the peripheral circulation (FIGURE 3)

The withdrawal of leukocytes from the peripheral circulation can also be initiated by the administration of histamine[2] It has been known for many years that the administration of histamine was associated with a leukopenia Similar leukopenias have been a common finding in marked allergic states or

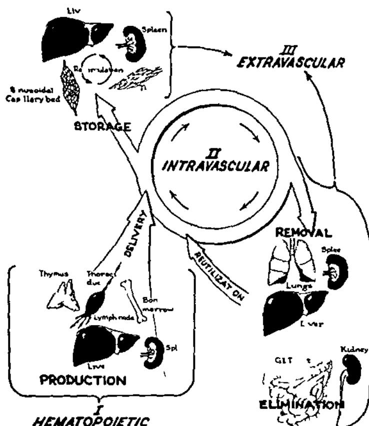


FIGURE 2 Th l f pa f the leukocyt d d d to th phases Th b und es t i ta t, a d a fr e flow of l kocyt f m n ph se t n th p b bl Altho gh t h be re llv c ced d that the predom na t flow s from I to III t sh ld b emphas zed th t the abil ty th phys ol g cal st te m y effect a leukocyt flow f m III to II II t I III to I

study, with the aid of venous and arterial catheterization techniques afforded an exceptional opportunity to explore the mechanisms involved which might also apply to the other removal sites It should be stressed that the lung is not the only organ involved in leukocyte removal and that it is not character istic of all removal sites[4] The emphasis upon the pulmonary circulation merely reflects the industry interest and singular devotion to this organ as the first removal site in a methodical approach to the total problem of leuko cyte physiology

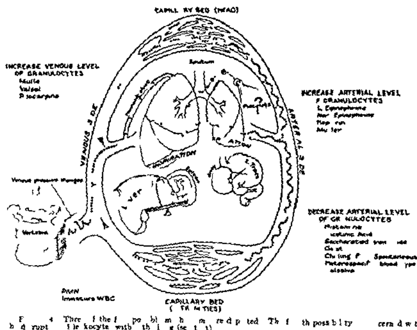
The peripheral blood leukocyte level has been known to fluctuate rapidly When samples of venous and arterial blood were obtained simultaneously from the pulmonary artery and the left ventricle aorta or a large peripheral artery the leukocyte level was found to be related to the phase and depth of the respiratory cycle[5] During the Mueller maneuver which is the physio logical counterpart of inspiration both the venous and arterial blood leukocyte

at other withdrawal sites The rate of removal of leukocytes in the pulmonary circulation in some instances exceeded 16 200 leukocytes per cubic millimeter per minute The granulocytes were almost exclusively, although not wholly, involved The total number of leukocytes involved is a function of the cardiac output in addition to the starting level of leukocytes time *etc* Although difficult to estimate the data suggest that large numbers of leukocytes can be sequestered in the pulmonary circulation within very short periods Surprisingly the control leukocyte level is reattained within 8 to 10 minutes after histamine administration in the nonleukemic subject but about 60 minutes following nicotinic acid

It becomes important therefore to determine the fate and destiny of those leukocytes trapped temporarily or otherwise in the pulmonary circulation At least four major possibilities exist (FIGURE 4)

(1) *The leukocytes are trapped transiently in the pulmonary circulation by margination and with each respiration equal numbers of leukocytes leave the pulmonary circulation to enter the arterial blood so that the count remains constant* The studies to date however show that fewer leukocytes leave the lung than enter

(2) *Some of the leukocytes trapped within the pulmonary circulation make their way through the pulmonary epithelium into the alveoli and then out through the sputum to be excreted to the exterior* Large numbers of granulocytes are commonly found in the alveoli without any evidence of infection Whether this is



F 4 Thre f the f po bl m h m re d p ted Th f th poss b l ty cern d w th b d rupt l le kocyte with th l g (se t t)

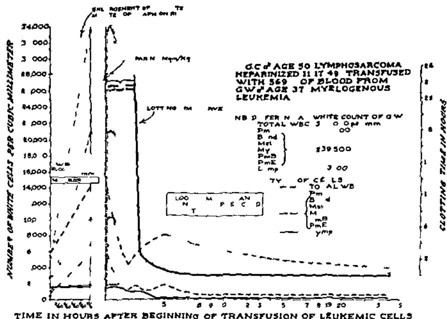
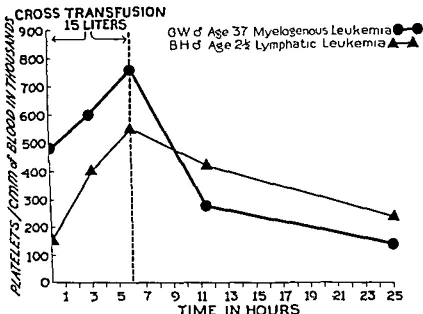


FIGURE 3 Desp t l tt g t me beyo d 24 hou d to hepa n 3 mgm./kg only b t 30 pe ent of th fused l kocyt s passed th lung ba r e Not th t m r of th lymo pho le s ame th ugh tha m m ture f m y t a la ge mber f the latt r we tra f d

anaphylaxis[8] It had always been assumed that such a leukopenia, following large amounts of histamine was caused by redistribution of the leukocytes attributed to pooling in the splanchnic circulation due to vasodilatation By utilizing simultaneous arterial and venous catheterization and isolating the pulmonary circulation between tips of the two sampling catheters it was possible to show that the administration of histamine in doses as small as 0 1 mgm intravenously will cause a prompt arterial leukopenia fully 60 to 120 seconds before the venous circulation reflects the arterial fall Similar findings will occur following the administration of nicotinic acid colloidal substances such as saccharated iron oxide plasmoid and gelatin or heterospecific blood In each instance, while the return to normal level usually occurred within a predictable time there was no evidence of overcompensation with a redelivery of such withdrawn leukocytes back into the peripheral circulation, although the possibility of course must be entertained that these trapped leukocytes were returned slowly to the circulation in numbers too small for one to detect accurately by counting methods In any event it is apparent that there are many dynamic changes in leukocyte number which are occurring from one moment to the next in the peripheral circulation

There appears to be a constant ebb and flow of leukocytes moving into and from the pulmonary circulation apparently associated with many voluntary and involuntary physiological processes This movement is reflected peripherally as a fluctuating leukocyte count The net result of this ebb and flow tide would appear to be a loss of leukocyte number in the lung and probably



F G 5 I re se pl t let mbe t l blood d g d ft a h e t f betwe patient w th gra locyt le k m a a d w th lymphocyt l k m

the leukocyte and that the leukocyte also contains large amounts of a thromboplastinlike material which is presumed to be responsible for margination when the circulation is slowed [12] It might be conjectured that platelets adherent to the surface of the leukocyte in the venous blood may be released in the lung or cytoplasmic fragments resulting from the disrupted leukocytes may appear in the arterial circulation as a plateletlike body which might account for the high counts recorded Whether these plateletlike bodies are in fact true platelets or merely appear to be morphologically similar remains to be determined Nevertheless this phenomenon might suggest a possible end result of some of the leukocytes and would account in part for the large turnover in leukocyte number the body appears to be continuously utilizing

It should be emphasized that the morphological appearance of the platelet under fixed techniques is different from that observed under phase microscopy and by supravital methods Furthermore there is no conclusive evidence at this time to substantiate whether platelets are manufactured in the lungs in the currently accepted fashion [?] from the megakaryocyte or merely stored there from other sites The lack of megakaryocytes in the human lung would discourage any serious consideration that platelets were manufactured in the pulmonary circulation from megakaryocytes Furthermore it is doubtful that the large mass of platelets that would be required to double or triple the platelet number in the circulation for prolonged periods of time could arise from the megakaryocyte in such a short time The reservoir of platelets in the pulmonary circulation is not large enough to supply this number of platelets

a major or insignificant route by which the leukocytes leave the lung has not been determined Weisberger *et al*[7] and Ambrus *et al*[8] confirmed the findings in man with tagged leukocyte studies in animals Many individuals swallow rather than bring up sputum for expectoration Ambrus *et al*[8] have found the gastrointestinal tract to be a second avenue of excretion of leukocytes

Studies of the sputum in patients with various types of the leukemias would indicate that granulocytes are present in fairly adequate numbers In patients with lymphocytic leukemia, while the peripheral count is almost exclusively lymphocytic the sputum usually contains large numbers of granulocytes

(3) *The leukocytes trapped within the pulmonary circulation make their way through the pulmonary epithelium into the interstitial tissues of the lung parenchyma and are picked up by the lymphatics to make their way via the thoracic duct to return to the venous circulation* Lymph constituents were studied following the canalization of the thoracic duct Profound histamine induced arterial leukopenias failed to show any increase in granulocyte number in the thoracic duct lymph in fact in some instances an absolute and relative decrease in leukocyte number occurred in both the thoracic duct lymph and the peripheral blood It would thus appear that the thoracic duct is not a significant route of escape for leukocytes from the pulmonary circulation under these circumstances

(4) *The fourth possibility remains in the hypothesis that at least some of the leukocytes undergo disintegration in the lung and make their way into the arterial circulation in forms that are not distinguishable as intact or identifiable leukocytes* At this point one must resort a bit to speculation and I should like to present a hypothesis which we have entertained for the past five years It should be clearly emphasized that the data at this time are only circumstantial and do not constitute definite proof nevertheless it is important that this possibility be postulated if only to discard it later in case proof is not forthcoming

In 1939 Howell and Donahue[9] proposed the lungs as a possible source of platelets in the dog and that platelets were manufactured in the pulmonary circulation by the megakaryocytes frequently found there These studies were not confirmed by Fidlar and Waters in 1941[10]

It was subsequently found that the administration of epinephrine to patients with a normal pulmonary circulation was followed by a marked arterial leukocytosis while the venous return to the lungs was essentially at control levels for at least one to two minutes thereafter[3] This arterial leukocytosis was associated with marked thrombocytosis also apparently emanating from the pulmonary circulation It was likewise observed on frequent occasions that large numbers of platelets appeared in the arterial circulation following the infusion of large numbers of leukocytes from patients with granulocytic leukemia Similarly there was a marked increase of platelet number in arterial blood in both participants following the initiation of a cross transfusion although both patients possessed low platelet levels[11] (FIGURE 5) It would appear therefore that the pulmonary circulation of man is capable of storing large numbers of leukocytes and platelets which could be released rapidly from the pulmonary circulation upon proper stimulation

It is known that the chemical constituency of the platelet is similar to that of

the leukocytosis that was observed from the pulmonary circulation The studies on the capacity of the spleen to deliver leukocytes back into the circulation to act as a reservoir for leukocyte number in man was performed in patients without obvious splenic abnormalities and therefore such interpretations can apply only to the normal spleen It should be emphasized moreover that such findings may be entirely different in the spleens of patients with various blood dyscrasia particularly hypersplenism

It has always been difficult to explain why the spleen is placed anatomically so that it receives arterial blood yet its drainage pours directly into the portal circulation for further transfer through the hepatic circulation a situation which seems to be reserved for organs primarily related to the processes of digestion A distinct relationship between the spleen and the liver has been often observed in Banti s syndrome and in situations related to portal hypertension Aside from portal hypertension however there are no distinct relationships between the spleen and the liver that have been previously reported

Splenectomies in the leukemias have been performed on many occasions with excellent results being reported in lymphocytic leukemias [15] Such good results however are not usually enjoyed in patients with granulocytic leukemia It has been possible to observe three cases (patients with granulocytic leukemia with splenectomy) in which careful studies could be made and nine similar situations were extracted from the literature [16]

A typical case was that of a 15 year old girl who had chronic granulocytic leukemia for three years At first X ray therapy apparently gave good results Subsequently however she proved more and more refractory to roentgen therapy As the disease progressed her spleen became larger and eventually filled the entire left abdomen causing great discomfort Splenectomy was entertained and despite our contrary advice it was performed Immediately after the splenectomy a leukocytosis occurred consisting primarily of immature forms in the peripheral blood which had rarely appeared before After a week or two however the leukocyte number and immature cell level fell but not quite to the original presplenectomy level Then despite repeated courses of X ray therapy the leukocyte count began to appear in the peripheral blood associated with an increase in the size of the liver Within a period of two months the liver had increased approximately four times the size it had been before the splenectomy Marked tenseness in the liver appeared with tenderness and constant discomfort of the patient Subsequently a marked progressive elevation in the leukocyte count was observed with a predominance of immature leukocytes The patient failed rapidly and died in myeloblastosis This picture was characteristic of two other patients who were observed and coincided with the nine other instances derived from the literature (FIGURE 6) While it is felt that these patients of course may not by any means be typical of all patients with granulocytic leukemia following splenectomy the course of events may merely reflect a peculiar form of the disease that was observed Nevertheless it demonstrated that the spleen was operating in tandem or in collaboration with the liver When the spleen was removed the entire burden of its activities may have been thrust upon the liver particularly in this instance the removal of leukocytes It can be speculated that as long as the

It seems more probable, therefore, that the apparent production of platelets in the lung could be related to some circulatory constituent At the present time the leukocyte must again be given serious consideration, and of the leukocytes the adult polymorphonuclear neutrophils are most suspect

This hypothesis should not in any way, be misconstrued as a denial of the formation of platelets from the megakaryocyte but rather suggests a possible additional method for the formation of circulating plateletlike bodies

Liver

Other organs of leukocyte sequestration act in a similar though less obvious pattern The liver appears to be an important leukocyte removal site although not as prodigious in capacity as the lung perhaps due to the fact that the lung has a greater blood flow traversing it With the development of the portal vein catheterization it has become possible to isolate the liver separately *in vivo* as has been done successfully with the pulmonary circulation Preliminary results indicate that the liver is not as efficient as the pulmonary circulation in either removal sequestration or delivery of leukocytes The liver poses a more complicated problem for study in that it has a dual arterial and venous inflow route Under the influence of histamine marked differences in leukocyte numbers between arterial or portal vein inflow samples have not been consistently found The quantitative relations of the fate and destiny of leukocytes in the liver of man remains to be determined although the boundaries have been defined

Spleen

The spleen has generally been considered as a potent leukocyte sequestration site It should be realized however that much of the experimental data upon which this premise has been based was obtained from studies on the dog Upon closer scrutiny of the anatomy of the human spleen it can be found that there are no active contractile muscular components of the splenic capsule in comparison to the heavy muscular coat of the spleen of the dog The spleen in man has proved to be not as efficient as the pulmonary circulation or the liver in the removal, storage or promptness of delivery of leukocytes back into the circulation

The leukocytosis observed following the administration of epinephrine associated with a prompt decrease in size of the spleen has been repeatedly employed as a test for estimating the capacity of the spleen as a leukocyte reservoir [13] Utilizing the simultaneous arterial and venous catheterization technique it was possible to administer epinephrine directly into the spleen in doses of 0 1 to 0 3 mgm within a period of 30 seconds [14] The administration of epinephrine in man by this route caused a prompt spasm of the splenic artery with contraction and an apparent decrease in blood flow through the arterial circulation of most of the spleen Shortly thereafter a marked release of leukocytes and platelets from the pulmonary circulation appeared in the peripheral blood Two to five minutes later a slow and gradual rise in leukocyte number appeared in samples from the hepatic vein which however never approached

kocytes in contrast to the pulmonary circulation which removes leukocytes both during direct infusion and also after stimulation by histamine

These findings of removal organs and the capacity for storage and prompt return into the circulation are merely reflections of the extremely dynamic hematological condition which exists in man Other data indicate that large numbers of leukocytes are being manufactured constantly and that an equivalent number of leukocytes is being removed from the circulation in an equal period of time if the total count remains constant There are apparently many areas of storage of large numbers of leukocytes in organs and in the tissue proper This extravascular pool is a prodigious one The intravascular and extravascular circulation are both actively and intimately related to the mechanisms for the destruction of leukocytes and reutilization of their products either for remanufacture of leukocytes building other tissues or in the general body metabolism Similarly various removal organs such as the lung via the putum gastrointestinal tract via its lumen the liver via the biliary system and the kidney via the ureters have direct access to the exterior for such products of leukocyte disintegration or intact leukocytes which summed up would make up a sizable route of excretion of leukocytes The mechanism of sequestration therefore apparently plays a purposeful role as an integral part of the physiology of the leukocyte in normal man and its equilibrium can be profoundly disturbed in certain disease states such as the leukemias

It is an engaging fact that the leukocyte level in the normal subject remains so constant from day to day Despite the many environmental influences which may cause physiological leukocytosis and a leukopenia the fluctuations are always transient and the daily mean leukocyte number remains remarkably constant This finding is not true of patients with any of the leukemias In the latter there is definite impairment of the rate and capacity for leukocyte removal or delivery and of the ability to maintain a rigid leukocyte level during or following the physiological stimuli which causes the leukocyte level to rise or fall There is no proof however that this impairment of the hematological hemostatic mechanism is the cause the result or merely coincidental to the leukemic process

The mechanisms for sequestration of leukocytes are complex and diverse as they are located in many sites throughout the body ill-defined as they are as yet in the mode of performance and delicately balanced as they are against one another all apparently for the single purpose of maintaining the hematological balance That this balance is disturbed in some blood dyscrasias namely the leukemias is evident The mechanism and the significance of the disturbance may be an important factor toward the eventual solution of this problem That possibility is for future work to decide

Summary

The hematological state in normal man has been characterized as an extremely variable yet dynamic equilibrium between the production and delivery of leukocytes their removal and their destruction In normal man this equilibrium is constantly undergoing changes to counteract the physiological alterations produced by many environmental and involuntary events The

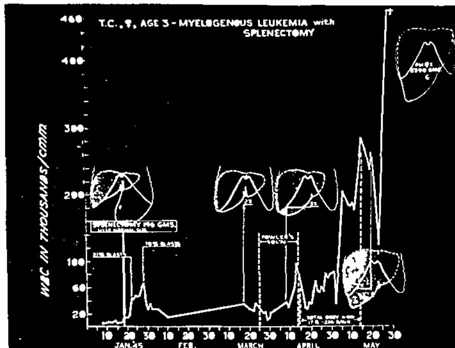


FIG. 6 C se of e nts 15 ye -old g l w th g locyt l kem a f llowi g spl t my N t th pr g s rease i ze f th l assoc t w th t nd cy tow d l kocyt s Th ma k d term l my l bla t y pa tly ppe d at th t m wh the l e ze re h d t m mum

liver could enlarge and maintain its capacity for withdrawal of leukocytes the peripheral leukocyte level did not rise appreciably As the liver reached its maximum size however its capacity for leukocyte removal was unable to increase any further and the mechanism for leukocyte removal decompensated as demonstrated by the gradual increase of immature forms in the peripheral blood which eventually led to demise of the patient with the peripheral blood almost completely myeloblastic This condition would suggest a coordinated effort of at least two of the entire group of removal organs in the apparent attempt of one to take over the function of another It is highly probable that all the removal organs and sites act in conjunction with one another

Capillaries in Striated Muscle

Studies on the vascular bed of an extremity have shown a capacity for removing leukocytes intermediate between that of the lung and the liver Under the influence of arterial infusion of histamine or gelatin the returning venous leukocyte count will decrease before the arterial count thus indicating a sequestration of leukocytes in the extremity The granulocytes are again primarily involved The perfusion of large numbers of leukocytes into the lower extremity however failed to demonstrate effective removal of leukocytes and the anticipated number was found in the venous blood [11] It would appear therefore, that the capillaries in striated muscle must be stimulated to remove leu

STUDIES ON LEUKOCYTIC SECRETORY ACTIVITY*

By Kenneth M Richter

Department of Anatomy University of Oklahoma School of Medicine Oklahoma City Okla

Introduction

The thought that leukocytes have a secretory function is not new (Renaut 1907 Ranvier cited by Renaut 1889 Metchnikoff 1905) In the past few decades the idea in some form has recurred periodically (Carrel 1922 1924 Carrel and Ebeling 1922a 1922b Sabin 1923 Kruchov 1945 Grand 1949) There is much evidence implicating the lymphocytes monocytes and plasma cells in the production of antibodies (Fagreus 1948 Burnet and Fenner 1949 Hartley 1940 Sabin 1938 1939 Dougherty Chase and White 1944 1945) and Burnet and Fenner have pointed out (1949) that the neutrophiles and eosinophiles have been neglected in discussions of antibody formation yet both need to be considered in any adequate account

The bulk of the evidence suggesting leukocytic secretory function has been indirect being based on physiologic data and accompanied by incomplete supplementary corroborative morphologic data yet both morphologic physiologic and chemical data seem essential to the ultimate elucidation of leucocytic secretory function Before going further into the question of leukocytic secretory activity *per se* it seems necessary therefore to consider briefly what are in typical glandular cells the established criteria of secretory activity the protoplasmic organelles most intimately concerned with secretory activity and the morphologic characteristics of intracellular secretion products

I *The Morphologic Characteristics of Secretory Activity in General*

It is beyond the scope of the present paper to attempt even a minor review of the abundant literature or even to trace the historical development of ideas bearing on the many facets of the secretory phenomenon For this the interested reader is referred to the very excellent reviews of Bowen (1929) Dawson (1942) Hibbard (1945) Hirsh (1939) Kirkman and Severinghaus (1938) and Renaut (1907) bearing on the cytology of glandular secretion It is admitted that important aspects of the secretory phenomenon are incompletely understood and in many respects unknown that points of view differ as to what does or does not constitute a secretion product or even secretory activity versus pathologic degeneration that it is not altogether evident where the line should be drawn even for purposes of discussion between secretory phenomena and those intracellular processes having to do with cell maintenance and differentiation phenomena of the cellular or protoplasmic units Nevertheless with a few doubtful exceptions as pointed out by Bowen (1929) and Dawson (1942) and others a few tangible morphologically discernible and physiologically demonstrable features have evolved which are characteristic of secretory activity in its entirety as it is manifest by exocrine glands and the majority of

* S ppo t d by gr t d f om th Am can C ce Soc ty pon re omm d t f th Comm tt G wth f th N t l R se h C un l d by g ts in d f om th H l H y Wh tney F und t

extremely stable leukocyte count in the peripheral circulating blood attests to the excellence of this balanced state In the leukemic state, this balance is impaired, at certain times, in favor of production and delivery and at other times, in favor of the removal and destruction within the hematopoietic sites the peripheral blood or the extravascular tissues The variability of the circulating life span of the leukocyte reflects these various states of imbalance as confirmed by the prolonged life spans of specific leukocyte types in the leukemias Data have also been presented to demonstrate the mechanisms of removal of leukocytes in normal and leukemic individuals under both physiological and pathological situations It has also been shown that certain leukemic patients at times are capable of withdrawing leukocytes from the peripheral circulation in an equally effective capacity as the normal subject

A hypothesis suggesting an additional method for the formation of platelets has been presented namely the formation of platelets from the granulocyte in the pulmonary circulation

It was also suggested that the sequestration of leukocytes in the various organs will differ with the various types of leukocytes in question and their maturity It is also apparent that despite the selectivity of various tissues for sequestration all the removal organs work in collaboration with one another for the removal of leukocytes from the peripheral circulation

References

1 Bierman H R 1949 Am Cancer Soc & Natl Cancer Inst Proc 1st Natl Cancer Conf 158–159

2 Bierman H R R L Byron Jr K H Kelly & L P White 1952 Some principles underlying the pathological physiology of the leukemias in man Am Cancer Soc & Natl Cancer Inst Proc 2nd Natl Cancer Conf

3 Bierman H R K H Kelly F L Cordes R L Byron Jr J A Polhemus & S Rappoport 1952 The release of leukocytes and platelets from the pulmonary circulation by epinephrine Blood 7 683–692

4 Lawrence J S 1941 Leukopenia J Am Med Assoc 116 478

5 Bierman H R K H Kelly F L Cordes N L Petrakis H Kass & E L Shpil 1952 The influence of respiratory movements upon the circulating leukocytes Blood 7 533–544

6 Webb R A 1924 The mechanism of anaphylactic leukopenia in dogs J Path Bact 27 79–94

7 Weisberger A S R W Heinle J P Storaasli & R Hannah 1950 Transfusion of leukocytes labelled with radioactive phosphorus J Clin Invest 29 336

8 Ambrus J L Personal communication

9 Howell W H & D D Donahue 1937 The production of blood platelets in the lungs J Exptl Med 65 177–204

10 Fidlar E & F T Waters 1941 The origin of platelets J Exptl Med 73 299–307

11 Bierman H R R L Byron K H Kelly K S Dod & P M Black 1951 Studies on cross circulation in man 1 Methods and clinical changes Blood 6 487–503

12 Vejlens G 1938 The distribution of leukocytes in the vascular system Acta Path Microbiol Scand Suppl 33

13 Frey W & S Lury 1914 Adrenalin zur funktionellen diagnostik der milz Untersuchungen auklinischen material Z ges exptl Med 2 50–64

14 Bierman H R R L Byron Jr & K H Kelly 1953 The role of the spleen in the leukocytosis following the intra arterial administration of epinephrine Blood 8 153–164

15 Fisher J H C S Welch & W Dameshek 1952 Splenectomy in leukemia and leukosarcoma New Engl J Med 246 477–484

16 Berlin R 1951 Red cell survival studies in normal and leukemic subjects Acta Med Scand 139 Suppl 252

STUDIES ON LEUKOCYTIC SECRETORY ACTIVITY*

By Kenneth M Richter

Department of Anatomy University of Oklahoma School of Medicine Oklahoma City Okla

Introduction

The thought that leukocytes have a secretory function is not new (Renaut 1907 Ranvier cited by Renaut 1889, Metchnikoff 1905) In the past few decades the idea in some form has recurred periodically (Carrel 1922 1924 Carrel and Ebeling 1922a 1922b Sabin 1923, Kruschov 1945 Grand 1949) There is much evidence implicating the lymphocytes monocytes and plasma cells in the production of antibodies (Fagreus 1948 Burnet and Fenner 1949 Hartley 1940 Sabin 1938 1939 Dougherty Chase and White 1944 1945) and Burnet and Fenner have pointed out (1949) that the neutrophiles and eosinophiles have been neglected in discussions of antibody formation yet both need to be considered in any adequate account

The bulk of the evidence suggesting leukocytic secretory function has been indirect being based on physiologic data and accompanied by incomplete supplementary corroborative morphologic data yet both morphologic physiologic and chemical data seem essential to the ultimate elucidation of leucocytic secretory function Before going further into the question of leukocytic secretory activity *per se* it seems necessary therefore to consider briefly what are in typical glandular cells the established criteria of secretory activity the protoplasmic organelles most intimately concerned with secretory activity and the morphologic characteristics of intracellular secretion products

I *The Morphologic Characteristics of Secretory Activity in General*

It is beyond the scope of the present paper to attempt even a minor review of the abundant literature or even to trace the historical development of ideas bearing on the many facets of the secretory phenomenon For this the interested reader is referred to the very excellent reviews of Bowen (1929) Dawson (1942) Hibbard (1945) Hirsh (1939) Kirkman and Severinghaus (1938) and Renaut (1907) bearing on the cytology of glandular secretion It is admitted that important aspects of the secretory phenomenon are incompletely understood and in many respects unknown that points of view differ as to what does or does not constitute a secretion product or even secretory activity versus pathologic degeneration that it is not altogether evident where the line should be drawn even for purposes of discussion between secretory phenomena and those intracellular processes having to do with cell maintenance and differentiation phenomena of the cellular or protoplasmic units Nevertheless with a few doubtful exceptions as pointed out by Bowen (1929) and Dawson (1942) and others a few tangible morphologically discernible and physiologically demonstrable features have evolved which are characteristic of secretory activity in its entirety as it is manifest by exocrine glands and the majority of

ppo t d by gr t d f om th Am C Soc ty po omm dat f th Comr tt
G owth f th N t l R se h C l d by g ts -d f om th H l H y Wh tney Fou dat

extremely stable leukocyte count in the peripheral circulating blood attests to the excellence of this balanced state In the leukemic state, this balance is impaired, at certain times, in favor of production and delivery and at other times, in favor of the removal and destruction within the hematopoietic sites the peripheral blood, or the extravascular tissues The variability of the circulating life span of the leukocyte reflects these various states of imbalance as confirmed by the prolonged life spans of specific leukocyte types in the leukemias Data have also been presented to demonstrate the mechanisms of removal of leukocytes in normal and leukemic individuals under both physiological and pathological situations It has also been shown that certain leukemic patients at times are capable of withdrawing leukocytes from the peripheral circulation in an equally effective capacity as the normal subject

A hypothesis suggesting an additional method for the formation of platelets has been presented, namely the formation of platelets from the granulocyte in the pulmonary circulation

It was also suggested that the sequestration of leukocytes in the various organs will differ with the various types of leukocytes in question and their maturity It is also apparent that, despite the selectivity of various tissues for sequestration all the removal organs work in collaboration with one another for the removal of leukocytes from the peripheral circulation

References

1 BIERMAN H R 1949 Am Cancer Soc & Natl Cancer Inst Proc 1st Natl Cancer Conf 158–159
2 BIERMAN H R R L BYRON JR K H KELLY & L P WHITE 1952 Some principles underlying the pathological physiology of the leukemias in man Am Cancer Soc & Natl Cancer Inst Proc 2nd Natl Cancer Conf
3 BIERMAN H R K H KELLY F L CORDES R L BYRON JR J A POLHEMUS & S RAPPOPORT 1952 The release of leukocytes and platelets from the pulmonary circulation by epinephrine Blood 7 683–692
4 LAWRENCE J S 1941 Leukopenia J Am Med Assoc 116 478
5 BIERMAN H R K H KELLY F L CORDES N L PETRAKIS H KASS & E L SHPIL 1952 The influence of respiratory movements upon the circulating leukocytes Blood 7 533–544
6 WEBB R A 1924 The mechanism of anaphylactic leukopenia in dogs J Path Bact 27 79–94
7 WEISBERGER A S R W HEINLE J P STORAASLI & R HANNAH 1950 Transfusion of leukocytes labelled with radioactive phosphorus J Clin Invest 29 336
8 AMBRUS J L Personal communication
9 HOWELL W H & D D DONAHUE 1937 The production of blood platelets in the lungs J Exptl Med 65 177–204
10 FIDLAR E & E T WATERS 1941 The origin of platelets J Exptl Med 73 299–307
11 BIERMAN H R R L BYRON K H KELLY K S DOD & P M BLACK 1951 Studies on cross circulation in man I Methods and clinical changes Blood 6 487–503
12 VEJLENS G 1938 The distribution of leukocytes in the vascular system Acta Path Microbiol Scand Suppl 33
13 FREY W & S LURY 1914 Adrenalin zur funktionellen diagnostik der milz Untersuchungen auklinischen material Z ges exptl Med 2 50–64
14 BIERMAN H R R L BYRON JR & K H KELLY 1953 The role of the spleen in the leukocytosis following the intra arterial administration of epinephrine Blood 8 153–164
15 FISHER J H C S WELCH & W DAMESHEK 1952 Splenectomy in leukemia and leukosarcoma New Engl J Med 246 477–484
16 BERLIN R 1951 Red cell survival studies in normal and leukemic subjects Acta Med Scand 139 Suppl 252

STUDIES ON LEUKOCYTIC SECRETORY ACTIVITY*

By Kenneth M Richter

Department of Anatomy University of Oklahoma School of Medicine Oklahoma City Okla

Introduction

The thought that leukocytes have a secretory function is not new (Renaut 1907 Ranvier cited by Renaut 1889 Metchnikoff 1905) In the past few decades the idea in some form has recurred periodically (Carrel 1922 1924 Carrel and Ebeling 1922a 1922b Sabin 1923 Kruschov 1945 Grand 1949) There is much evidence implicating the lymphocytes monocytes and plasma cells in the production of antibodies (Fagreus 1948 Burnet and Fenner 1949 Hartley 1940 Sabin 1938 1939 Dougherty Chase and White 1944 1945) and Burnet and Fenner have pointed out (1949) that the neutrophiles and eosinophiles have been neglected in discussions of antibody formation yet both need to be considered in any adequate account

The bulk of the evidence suggesting leukocytic secretory function has been indirect being based on physiologic data and accompanied by incomplete supplementary corroborative morphologic data yet both morphologic physiologic and chemical data seem essential to the ultimate elucidation of leucocytic secretory function Before going further into the question of leukocytic secretory activity *per se* it seems necessary therefore to consider briefly what are in typical glandular cells the established criteria of secretory activity the protoplasmic organelles most intimately concerned with secretory activity and the morphologic characteristics of intracellular secretion products

I *The Morphologic Characteristics of Secretory Activity in General*

It is beyond the scope of the present paper to attempt even a minor review of the abundant literature or even to trace the historical development of ideas bearing on the many facets of the secretory phenomenon For this the interested reader is referred to the very excellent reviews of Bowen (1929) Dawson (1942) Hibbard (1945) Hirsh (1939) Kirkman and Severinghaus (1938) and Renaut (1907) bearing on the cytology of glandular secretion It is admitted that important aspects of the secretory phenomenon are incompletely understood and in many respects unknown that points of view differ as to what does or does not constitute a secretion product or even secretory activity versus pathologic degeneration that it is not altogether evident where the line should be drawn even for purposes of discussion between secretory phenomena and those intracellular processes having to do with cell maintenance and differentiation phenomena of the cellular or protoplasmic units Nevertheless with a few doubtful exceptions as pointed out by Bowen (1929) and Dawson (1942) and others a few tangible morphologically discernible and physiologically demonstrable features have evolved which are characteristic of secretory activity in its entirety as it is manifest by exocrine glands and the majority of

* S ppo t d by gr t d f om th Am an C ce Soc ty upo re mm dat f th Comm tt Growth f th N t l R se h C un l d by gr ts in d from th H len H y Wh t y Foundat on

extremely stable leukocyte count in the peripheral circulating blood attests to the excellence of this balanced state In the leukemic state, this balance is impaired, at certain times, in favor of production and delivery and, at other times in favor of the removal and destruction within the hematopoietic sites the peripheral blood, or the extravascular tissues The variability of the circulating life span of the leukocyte reflects these various states of imbalance as confirmed by the prolonged life spans of specific leukocyte types in the leukemias Data have also been presented to demonstrate the mechanisms of removal of leukocytes in normal and leukemic individuals under both physiological and pathological situations It has also been shown that certain leukemic patients, at times are capable of withdrawing leukocytes from the peripheral circulation in an equally effective capacity as the normal subject

A hypothesis suggesting an additional method for the formation of platelets has been presented namely, the formation of platelets from the granulocyte in the pulmonary circulation

It was also suggested that the sequestration of leukocytes in the various organs will differ with the various types of leukocytes in question and their maturity It is also apparent that, despite the selectivity of various tissues for sequestration, all the removal organs work in collaboration with one another for the removal of leukocytes from the peripheral circulation

References

1 BIERMAN H R 1949 Am Cancer Soc & Natl Cancer Inst Proc 1st Natl Cancer Conf 158–159
2 BIERMAN H R R L BYRON JR K H KELLY & L P WHITE 1952 Some principles underlying the pathological physiology of the leukemias in man Am Cancer Soc & Natl Cancer Inst Proc 2nd Natl Cancer Conf
3 BIERMAN H R K H KELLY F L CORDES R I BYRON JR J A POLHEMUS & S RAPPOPORT 1952 The release of leukocytes and platelets from the pulmonary circulation by epinephrine Blood 7 683–692
4 LAWRENCE J S 1941 Leukopenia J Am Med Assoc 116 478
5 BIERMAN H R K H KELLY F L CORDES N L PETRAKIS H KASS & E L SHPIL 1952 The influence of respiratory movements upon the circulating leukocytes Blood 7 533–544
6 WEBB R A 1924 The mechanism of anaphylactic leukopenia in dogs J Path Bact 27 79–94
7 WEISBERGER A S R W HEINLE J P STORAASLI & R HANNAH 1950 Transfusion of leukocytes labelled with radioactive phosphorus J Clin Invest 29 336
8 AMBRUS J L Personal communication
9 HOWELL W H & D D DONAHUE 1937 The production of blood platelets in the lungs J Exptl Med 65 177–204
10 FIDLAR E & E T WATERS 1941 The origin of platelets J Exptl Med 73 299 307
11 BIERMAN H R R L BYRON K H KELLY K S DOD & P M BLACK 1951 Studies on cross circulation in man 1 Methods and clinical changes Blood 6 487–503
12 VEJLENS G 1938 The distribution of leukocytes in the vascular system Acta Path Microbiol Scand Suppl 33
13 FREY W & S LURY 1914 Adrenalin zur funktionellen diagnostik der milz Untersuchung n auklinischen material Z ges exptl Med 2 50–64
14 BIERMAN H R R L BYRON JR & K H KELLY 1953 The role of the spleen in the leukocytosis following the intra arterial administration of epinephrine Blood 8 153–164
15 FISHER J H C S WELCH & W DAMESHEK 1952 Splenectomy in leukemia and leukosarcoma New Engl J Med 246 477–484
16 BERLIN R 1951 Red cell survival studies in normal and leukemic subjects Acta Med Scand 139 Suppl 252

STUDIES ON LEUKOCYTIC SECRETORY ACTIVITY*

By Kenneth M Richter

Department of Anatomy University of Oklahoma School of Medicine Oklahoma City Okla

Introduction

The thought that leukocytes have a secretory function is not new (Renaut 1907, Ranvier cited by Renaut 1889 Metchnikoff 1905) In the past few decades the idea in some form has recurred periodically (Carrel 1922 1924 Carrel and Ebeling 1922a 1927b Sabin 1923 Kruschov 1945 Grand 1949) There is much evidence implicating the lymphocytes monocytes and plasma cells in the production of antibodies (Fagreus 1948 Burnet and Fenner 1949 Hartley 1940 Sabin 1938 1939 Dougherty Chase and White 1944 1945) and Burnet and Fenner have pointed out (1949) that the neutrophiles and eosinophiles have been neglected in discussions of antibody formation yet both need to be considered in any adequate account

The bulk of the evidence suggesting leukocytic secretory function has been indirect being based on physiologic data and accompanied by incomplete supplementary corroborative morphologic data yet both morphologic physiologic, and chemical data seem essential to the ultimate elucidation of leucocytic secretory function Before going further into the question of leukocytic secretory activity *per se* it seems necessary therefore to consider briefly what are in typical glandular cells the established criteria of secretory activity the protoplasmic organelles most intimately concerned with secretory activity and the morphologic characteristics of intracellular secretion products

I *The Morphologic Characteristics of Secretory Activity in General*

It is beyond the scope of the present paper to attempt even a minor review of the abundant literature or even to trace the historical development of ideas bearing on the many facets of the secretory phenomenon For this the interested reader is referred to the very excellent reviews of Bowen (1929) Dawson (1942) Hibbard (1945) Hirsh (1939) Kirkman and Severinghaus (1938) and Renaut (1907) bearing on the cytology of glandular secretion It is admitted that important aspects of the secretory phenomenon are incompletely understood and in many respects unknown that points of view differ as to what does or does not constitute a secretion product or even secretory activity versus pathologic degeneration that it is not altogether evident where the line should be drawn even for purposes of discussion between secretory phenomena and those intracellular processes having to do with cell maintenance and differentiation phenomena of the cellular or protoplasmic units Nevertheless with a few doubtful exceptions as pointed out by Bowen (1929) and Dawson (1942) and others a few tangible morphologically discernible and physiologically demonstrable features have evolved which are characteristic of secretory activity in its entirety as it is manifest by exocrine glands and the majority of

* S pport d by gra t id f om th Am ca Cance Soc ty pon re omm nd t f the Comm ttee G owth f th N t al R se h Cou l d by grants-in id f om the H le H y Wh tney Foundation

endocrine glands Secretory activity, no matter how broadly or how narrowly it is considered, (1) is a process of living cells, (2) it involves a morphologically discernible phase of elaboration of a product, (3) it involves a morphologically variable but discernible phase of accumulation and maturation of product, (4) it involves the discharge or some other kind of removal of the product from the cell and (5) it is cyclic in character this cycle being passed through just once as in the case of the holocrine gland cells or, repetitively, as in the case of the merocrine and apocrine types of glandular cells (Greep 1954 Dawson, 1942 Bowen 1929, 1926, 1924 F Schultze, 1867 Renaut 1907, and others)

It is therefore essential in any discussion of leukocytic secretory activity to consider the morphology of secretion products Although other workers had described various intracellular secretion products Renaut (1907) was the first to attempt to systematize secretory activity and to characterize its products While his interpretations were conjectural in part and while he erred in certain instances as to the mode of origin growth and identity of the products his general descriptive treatment of the products is classical and is equally applicable today as then Renaut recognized three distinct types of secretory activity each characterized by a morphologically distinct type of secretion product The first and most simple type he termed plasmocrine It is characterized by the elaboration and growth of homogeneous fluid droplets or vacuoles which he thought to be basically comprised of water and salts (exemplified by thyroid and pituitary cells D Angelo 1941 Payne 1940, parathyroid De Robertis 1940) The second type, rhagiocrine (exemplified by the majority of exocrine glands, such as the pancreas, parotid and lachrymal glands Bowen 1929) is characterized by the formation and growth of vacuolar or fluid droplets containing grains of segregation of variable character from one species of cell to another which during growth and maturation, could completely fill the vacuolar component (Payne 1940 granular signet ring vacuoles) Renaut s third type the lipocrine is characterized by the formation and growth of simple discrete fat or lipoid droplets at certain points in the cell (Bennett 1940 adrenal Bowen 1929 Renaut 1907) It is significant that Renaut recognized that all combinations of these types could occur simultaneously in the same cell (Payne 1940) While it has been claimed that all secretory products may not be morphologically discernible within the cell this claim is generally considered to be unlikely (Bowen 1929) and due to incompleteness of data (Dawson 1942) It is possible that the cytoplasmic sloughing of mononuclears described by Downey (1913) Sabin (1938 1939) and Dougherty Chase and White (1944) and possibly by Engelbert (1953) may be exemplary of specific products which are not morphologically discernible within the cell although Sabin did not consider such sloughed off bits of cytoplasm as a secretion (1939) In these instances it would seem that further precise cytologic studies are indicated It is the plasmocrine type which most generally concerns us here in the question of leukocytic secretory activity

A sixth feature of the secretory phenomenon stated broadly is that secretory activity is accompanied by morphologic change in protoplasmic organelles or components other than the product although which organelles change and the extent and character of such changes are variable from one s cies of cell

another (Bowen 1929 Dawson, 1942 Severinghaus 1933 Saguchi 1920 Hirsch 1939 Hibbard 1945 Gatenby 1931 Lavdovsky, 1877 Caspersson 1947) While no one would deny that secretory activity must depend upon the combined integrated functioning of all parts of the cell *e g* nucleus nucleoli mitochondria golgi complex cytoplasmic ground substance protoplasmic membranes *etc* it has not been possible so far to define clearly the particular role of each cellular component if any in the various phases of the secretory phenomenon It has to be admitted that the vast bulk of experimental physiologic and morphologic data implicate the golgi complex as being most intimately concerned in the secretory phenomenon (Bowen 1929 1927 1926 1925 Baker 1950 1951 Uhlenhuth 1934 B I Baker 1942 Worley 1944a 1944b D Angelo 1941 Hirsch 1939 Kirkman and Severinghaus 1938 Hibbard 1945 Gatenby 1931 1951) although precisely how is in the main conjectural At the same time other evidence indicates that nucleoli (Caspersson 1947 1950) and mitochondria (Haurowitz 1953) are also involved

II *Status of the Nucleolar System in Leucocytes*

The nucleolar system for many years has been thought to be involved somehow in secretory activity either directly (Bowen 1929) or indirectly via the involvement of nucleic acids in protein synthesis (Caspersson 1947 1950 and others) According to the Caspersson mechanism (1947 1950 Thorell 1944) a reduced or absent nucleolar system is indicative of reduced protein synthesis whether it be associated with growth or secretion

The greater part of the workers are in general agreement that the majority of all varieties of lymphocytes except the small variety generally possess nucleolar material (Downey 1930 Hall 1938 Bloom 1938 Richter 1942) In the granular leukocytes, it is generally agreed that the immature forms myeloblasts through myelocytes possess a well-developed system of large nucleoli of characteristic form and number (FIGURES 2 to 4) (Butterfield 1907 and Schridde 1907 cited by Thorell 1944 Brugsch and Schilling 1908 Naegeli 1931 Downey 1928 1930 Noel and Pigeaud 1930 Richter 1942) Mature granulocytes also have a well-developed nucleolar system which differs from that of the immature forms in being dispersed as small granules imbedded in the chromatin masses and it has been shown (Richter 1942 Parrisius and Schlopsnies 1927 Schilling 1929) in human circulating neutrophiles under various experimental conditions that the dispersed granular nucleoli could undergo aggregation and fusion to reconstitute nucleoli which in number size shape and staining reaction are identical with those occurring in the immature forms and myeloblasts Thorell (1944) in studies of the Caspersson mechanism in differentiating granulocytes shows U V absorption photomicrographs of the nucleoli in mature and immature granulocytes in the rat which are precisely like those described previously (Richter 1942) Curiously however he interpreted his findings as indicating a reduction and final disappearance of nucleoli during the maturation of granulocytes (FIGURES 8 22 23)

In view of the evidence it seems possible to conclude only that the majority of the leukocytes with the possible exception of the small variety of lymphocytes have a well developed and characteristic nucleolar system

endocrine glands Secretory activity, no matter how broadly or how narrowly it is considered (1) is a process of living cells, (2) it involves a morphologically discernible phase of elaboration of a product (3) it involves a morphologically variable but discernible phase of accumulation and maturation of product, (4) it involves the discharge or some other kind of removal of the product from the cell and (5) it is cyclic in character, this cycle being passed through just once as *in the case of the holocrine gland cells or repetitively, as in the case of the* merocrine and apocrine types of glandular cells (Greep 1934, Dawson 1942, Bowen 1929 1926, 1924 F Schultze, 1867, Renaut 1907, and others)

It is therefore essential in any discussion of leukocytic secretory activity, to consider the morphology of secretion products Although other workers had described various intracellular secretion products Renaut (1907) was the first to attempt to systematize secretory activity and to characterize its products While his interpretations were conjectural in part and while he erred in certain instances as to the mode of origin growth and identity of the products, his general descriptive treatment of the products is classical and is equally applicable today as then Renaut recognized three distinct types of secretory activity each characterized by a morphologically distinct type of secretion product The first and most simple type he termed plasmocrine It is characterized by the elaboration and growth of homogeneous fluid droplets or vacuoles which he thought to be basically comprised of water and salts (exemplified by thyroid and pituitary cells D Angelo 1941, Payne 1940, parathyroid De Robertis 1940) The second type, rhagiocrine (exemplified by the majority of exocrine glands such as the pancreas, parotid and lachrymal glands Bowen 1929) is characterized by the formation and growth of vacuolar or fluid droplets containing grains of segregation of variable character from one species of cell to another which during growth and maturation could completely fill the vacuolar component (Payne 1940 granular signet ring vacuoles) Renaut s third type the lipocrine is characterized by the formation and growth of simple discrete fat or lipoid droplets at certain points in the cell (Bennett 1940, adrenal Bowen 1929 Renaut, 1907) It is significant that Renaut recognized that all combinations of these types could occur simultaneously in the same cell (Payne 1940) While it has been claimed that all secretory products may not be morphologically discernible within the cell this claim is generally considered to be unlikely (Bowen 1929) and due to incompleteness of data (Dawson 1942) It is possible that the cytoplasmic sloughing of mononuclears described by Downey (1913) Sabin (1938 1939) and Dougherty Chase and White (1944) and possibly by Engelbert (1933) may be exemplary of specific products which are not morphologically discernible within the cell although Sabin did not consider such sloughed off bits of cytoplasm as a secretion (1939) In these instances it would seem that further precise cytologic studies are indicated It is the plasmocrine type which most generally concerns us here in the question of leukocytic secretory activity

A sixth feature of the secretory phenomenon stated broadly is that secretory activity is accompanied by morphologic change in protoplasmic organelles or components other than the product although which organelles change, and the extent and character of such changes are variable from one species of cell to

These two components may (1) be morphologically distinguishable side by side within the same golgi structure (Uhlenhuth 1934 Richardson 1934 Hertwig 1929 Dalton 1953, Worley 1944 1946 Richter 1940 1942) or (2) so associated as to be spatially indistinguishable from each other within the same golgi structure (Richter 1938 1940 Hirsch 1939 Kirkman and Severinghaus 1938) or (3) as completely separate morphologically discrete bodies (Richter 1938 1940 1942 Pantel and De Sinety 1906, Payne 1927) There is evidence showing various intergradations between these three types (Richter 1940)

In the *lymphocytes* and by means of classical golgi techniques the osmiophile golgi substance has been described variously as granules oriented about the centriole (Ito Takahashi and Mizutani 1938 Maximow 1928a) or as a small compact reticulum eccentrically located near the nucleus (Deineka 1912, Cowdry 1921 von Bergen 1904 Richter 1942) In the larger varieties of lymphocytes the osmiophile golgi substance comprises more than a single compact reticular or vesicular structure there being several golgi bodies variously disposed about the nucleus (Estable 1931 Richter 1942 Ehrich 1934) The osmiophile golgi substance also has been shown to enclose a central osmiophobe golgi mass in these cells (Dawson and Spark 1929 Estable 1931 Richter 1942) This duplex and essentially vesicular character of the golgi complex in the lymphocytes can be demonstrated in the living cells by phase contrast microscopy (FIGURES 1 4) (Richter, 1951 1952 Bessis 1949b)

The golgi complex in the *monocytes* as demonstrated by classical golgi techniques (Ehrich 1934 Dawson and Spark 1929) and by phase contrast microscopy (Richter 1951 1952a 1952b Hoffmann and Rottino 1950) is not essentially different from that of the large varieties of lymphocytes (FIGURE 10)

In general it would seem that in the lymphocytes and monocytes the golgi complex represents a system of the above mentioned first type in which the classical osmiophile and osmiophobe components are morphologically or spatially distinguishable within the same golgi structure of which there may be one or several per cell depending upon the cell species and variety

In the *eosinophiles* according to Cowdry (1921) certain of the classical golgi techniques sometimes revealed a small juxtanuclear osmiophilic golgi structure More frequently it was the specific eosinophilic granules which were revealed and this showing was true also for the basophiles in which a juxtanuclear net was not seen Dawson and Spark s findings (1929) were similar In unpublished studies of my own on a case of eosinophilic leukemia it was also the eosinophilic granules which were revealed by the classical golgi techniques The granules generally showed a central osmiophobic mass and an enclosing osmiophilic component The same duplex character is shown by the eosinophilic granules in living cells by dark contrast medium phase microscopy (FIGURE 5) (Bessis 1949a) after staining by Sudan black B (Bloom and Wislocki 1950) and by elctron microscopy (Aleksandrowicz Blicharski and Feltynowski 1952) It would seem on the basis of the evidence obtained generally by classical golgi methods and confirmed by histochemical methods and by phase microscopic studies of the living cells that there is no consistently demonstrable osmiophilic juxtanuclear golgi structure present, in the eosinophiles

It is interesting, in this connection, to note that Kossel (1894, cited by Metchnikoff, 1905) expressed the view that nucleic acid was secreted by the cell nucleus and accumulated in the vacuoles of the phagocytic leukocytes He also reported a bactericidal effect of nucleic acid LaCour (1944) and Discombe (1946) have described the extrusion of heterochromatin granules from the nuclei of mature granulocytes Koller (1947) has suggested that this expulsion may well be a mechanism of maintaining the high nucleic acid content of the cytoplasm of these cells

III *Mitochondrial Content of Leukocytes*

Brief mention should be made of the mitochondrial content of the several species of leukocytes especially so in view of the possible role of these bodies in protein synthesis and in the synthesis of antibodies (Haurowitz 1953, Crampton and Haurowitz, 1950 and others) and to the secretory phenomenon generally

Mitochondria are present in the mononuclears as short rods sometimes granules, or filaments whether visualized supravitally by Janus green by phase microscopy (FIGURE 10) or conventional mitochondrial preparations of fixed cells (Maximow 1909 1928 Schridde 1907 Butterfield, Heineke and Meyer 1909, Cunningham, Sabin and Doan 1925 Bloom, 1938, Richter 1942 1951 1952a 1952b Dubreuil 1913)

In the granulocytes mitochondria are generally considered to be present in the immature forms as short rods (Butterfield Heineke, and Meyer, 1909, Downey, 1928 Schilling 1929, and others) In the mature forms the mitochondria have been reported to be reduced in number and sometimes lacking, although the majority of workers regularly identify them in supravital preparations (Cowdry 1914 Bunting 1938 Cunningham and Tompkins 1930 Tompkins and Cunningham 1938 Sabin 1923 Schwind, 1950 Richter, 1942)

It would seem that all varieties of leukocytes normally contain mitochondria, but that the number varies from one cell species to another

IV *The Status of the Golgi Complex in Leukocytes*

The golgi complex ever since its discovery has been a subject of such heated controversy (Palade and Claude 1949a 1949b Bensley 1910 1951 Gatenby 1931 1951 Bowen 1929a 1929b Worley 1944a 1944b 1951 Hirsch 1939 Nassonov 1923 Dalton 1953 Parat and Painleve 1925 Gresson 1952 Baker 1944–1945) that I approach a discussion of it in the leukocytes with considerable reluctance

Descriptively only and without any connotation as to the chemical composition of the golgi complex the bulk of the past and current literature supports the idea that this complex generally is of a duplex structure being comprised of an osmiophilic or argentophilic component (the chromophile of Worley the apparate externum of Hirsch the dense lipoid containing substance of Baker) and an osmiophobic or argentophobic component (the idiosome or idiosomal part of Bowen the chromophobe of Worley the apparate internum of Hirsch the diffuse lipoid containing substance of Baker Hirsch 1939 Bowen 1929 1926 1925 Worley 1946 1951 Meves 1896 Baker 1944–1945 Sosa 1949)

(FIGURES 1, 4) the osmiophilic and osmiophobic components separate from each other at the beginning of the growth phase to form a single discrete osmiophilic body and a discrete osmiophobic body The former differentiates into a juxta nuclear and, finally a perinuclear osmiophilic structure The osmiophobic body through a series of alternating growth and constriction processes, gives rise to numerous small osmiophobic corpuscles widely distributed in the general cytoplasm Fourth it seems pertinent as demonstrating a possible common cytophysiologic bond between the specific granules and the golgi complex to note as determined by U V fluorescence microscopy that the carcinogenic hydrocarbons benzpyrene dibenzanthracene and methylcholanthrene are especially localized by living leukocytes in the specific granules of the granulocytes and in the golgi complex of the mononuclears and that their distribution in these structures seems to coincide well with the osmiophobic component in each instance (FIGURES 2 3 and 27 28 Richter, 1952) The same thing is true of other tissue cell types which I have examined Fifth Wallgren in a most interesting series of papers (1951 1946a 1946b) relates the specific granules to a fundamental double-droplet cytoplasmic structural system This system as he has observed it by dark field illumination in the living granulocytes (particularly evident in the eosinophiles), consists of (1) dark droplets which he recognizes as specific granules and of (2) luminous (pale) smaller droplets which he recognizes as (3) tiny vacuoles imbedded in a pale slightly luminous matrix or hyaloplasm Wallgren describes precisely the same system as comprising the golgi complex of the normoblast megaloblast plasma cell and lymphocytes It seems certain in the case of the eosinophiles and mononuclears that Wallgren s dark droplets coincide well with the osmiophobic component, and that his slightly luminous matrix coincides with the osmiophilic component of these structures Wallgren has described the matrical component of the golgi complex in mononuclears as changing shape rapidly and of extending processes into the cytoplasm I have recorded similar changes in configuration of the perinuclear osmiophilic golgi component in living neutrophiles by cinematography in combination with the phase microscope (FIGURES 22 23) It is significant to note here in connection with the neutrophile as recorded by motion pictures that the only formed components which are seen to be chacteristically associated with the perinuclear osmiophilic golgi structure (equivalent to Wallgren s matrix or hyaloplasm) are the specific neutrophilic granules

It seems necessary in view of the evidence to suggest that in the neutrophile the specific granules functionally and morphologically are equivalent to the conventional osmiophobic golgi component and that in these cells the two components are present as morphologically separate and discrete structural systems, as in the third type of golgi complex mentioned earlier This suggestion should not be taken to mean that the two components are without functional interdependance or that during the normal course of events they do not get together for it can be demonstrated in the living neutrophiles that the specific granules in the course of cytoplasmic streaming do establish contacts with the perinuclear osmiophile golgi structure

Cytoplasmic vacuoles or droplets (known variously as secretory vacuoles

certainly, and possibly in the basophile also and that, when this structure is revealed, its showing is due to a clumping of a few impregnated eosinophilic granules in the region of the cytocentrum, as suggested by Dawson and Spark (1929)

In view of the circumstances, it seems necessary to suggest that the eosinophilic granules collectively comprise an extensive but disperse type of golgi complex in which each granule is constituted of an osmiophilic and an osmiophiophobic part in a manner precisely like that obtaining in the several varieties of lymphocytes

In the *neutrophiles* several workers have demonstrated osmiophilic golgi substance by classical golgi methods (von Bergen 1904, Cowdry, 1921, Estable 1931, Ehrich 1934, Richter 1942) The bulk of the evidence indicates that while there is in the mature neutrophile a small juxtanuclear structure in the region of the nuclear concavity it is also continuous as a much attenuated complete perinuclear structure most intimately conforming to the shape of the nuclear segments (FIGURE 6) It has been shown, too, that under certain circumstances the entire perinuclear structure may undergo confluence and form a single compact osmiophilic structure eccentrically situated on or about the nucleus (Richter 1942) see FIGURES 7, 9 This structure is also demonstrable in the living cell with the phase microscope as a somewhat clear, irregularly attenuated juxtanuclear or perinuclear mass (FIGURES 22 23 30) The latter structure also has been observed by Bessis (1949a) although he did not recognize it as such No osmiophobe substance has been described in this particular structure

Some workers in the past have considered the neutrophilic granules (as well as the eosinophilic and basophilic granules) variously as special secretory products (Renaut 1889 Bunting 1928 Noel and Pigeaud 1930) as derivatives somehow of mitochondria or as of extracellular origin (Bunting 1928 Sabin, Austrian Cunningham and Doan 1924) At this time the facts seem not to warrant these interpretations It was suggested (Richter 1942) on the basis of certain cytophysiologic considerations and others that the neutrophilic granules of the polymorph may be fundamentally equivalent to the osmiophobic component of the lymphocytic golgi complex and to that of other tissue cells First there are mitochondria present in these cells even though they are reduced in number (Richter 1942 Bloom and Wislocki 1950 Cowdry 1914 Tompkins and Cunningham 1938 Schwind 1950 Dawson and Spark 1929 Hall 1938 1931 and others) Second the specific granules during their genesis first appear in the region of the myeloblastic golgi complex as reported by Ehrich (1934) Simpson and Deming (1927) Maximow and Bloom (1930) Schilling (1929) Third the golgi complex of the myeloblast is clearly visible with the phase microscope as a discrete juxtanuclear body (Creep 1954) Although the precise details on this point are much in need of clarification the genesis of the specific neutrophilic granules and of the perinuclear osmiophilic golgi structure and their relations to each other in the living cell are strikingly like those shown by the golgi complex during spermatogenesis in *Notonecta* (Pantel and De Sinety 1906 Richter 1940) In *Notonecta* briefly put and starting with a golgi body precisely like that occurring in the small lymphocytes

to measure and it certainly would not be what one could call obvious Exemplary of the point made is the fact that the vacuoles when first discernible in the living leukocytes are smaller than or just about the same size as the specific granules or golgi bodies associated with them (Sabin 1923 Simpson 1921, Wallgren 1951) I have confirmed this finding by cinematography (FIGURE 33)

In summary of the data on the formation of cytoplasmic vacuoles in the leukocytes, it seems necessary to suggest that vacuoles arise in the leukocytes just as in the majority of other tissue and glandular cell types in association with the golgi complex and may be considered as a functional expression of it

V *Observations on an Apparently True Type of Merocrine and Plasmocrine Secretory Activity by Several Species of Leukocytes*

Material and methods For these studies the circulating leukocytes of human peripheral blood were used By aseptic technique throughout small drops of whole blood obtained by finger puncture were placed on physiologically clean microscope slides and covered quickly with equally clean No 1 coverslips The latter were then quickly sealed around the edges with sterile petrolatum to prevent contamination and desiccation of the spread film of blood Such preparations were examined microscopically at either room or body temperatures in a special constant temperature incubator housing the microscope

Observations were made with the phase contrast microscope employing dark-contrast medium and B minus phase objectives The former phase objectives were most satisfactory The light source was a Mikrark illuminator equipped with a 100 w zirconium arc bulb A special Corning infrared absorbing filter No 3966 and a green filter of unknown composition were used with the illuminator Cinematographic records were made with 16 mm Bolex and Cine Special cameras in combination with Bausch and Lomb cinematographic accessories All motion picture records were made at eight frames per second

Observations When preparations are set up in the manner described a survey of the entire blood film after a lapse of 30 or more minutes reveals that many but not all of the leukocytes namely the neutrophiles eosinophiles and monocytes contain one or more prominent clear vacuolar droplets of variable size and distribution in the cytoplasm They are discernible in the living unstained cell with the phase microscope (FIGURES 15 16 17) with the ordinary light microscope and with the dark field microscope (M Schultze 1865 Renaut 1889 Metchnikoff 1905 Jordan 1925 Wallgren 1951 1946a 1946b Bessis 1949b Richter 1952a 1952b 1954 1951) With dark medium phase contrast the droplets appear as discrete milky white spherical bodies (FIGURES 15 16) They color somewhat metachromatically in the living cell with the supravital dye neutral red (Rosin and Biebergeil 1904 Metchnikoff 1905 Renaut 1907 Arrigoni 1908 Dubreuil 1913 Shipley 1919, Simpson 1921 Sabin 1923, Maximow 1927 1928a b c Rhoads and Parker 1928 Dawson and Spark 1929 Wilson and Cunningham 1929 Bloom 1928a 1928b Hall 1931 Lewis and Rubin 1932 Tompkins and Cunningham 1938 Lawrence and Todd 1941 Schwind, 1950 Jackson 1954) It is evident that these droplets have been known to us for a long time However due to differences in

acrosomal vesicles segregation vacuoles degeneration vacuoles) have been the object generally of much study (von Mollendorff 1937, 1938 Kedrowski 1932 1934, 1935a, 1935b Dustin 1941, 1944, 1947, Heinz, 1890 Gurber 1890 M Schultz, 1865, Lettre and Albrecht, 1943, Renaut, 1907) These vacuoles have been tied variously into the problem of the golgi complex, for example, as being products of golgi activity or as representing the essential structure of the golgi complex, or some part only of it (Bowen, 1929, 1927 Baker 1944–1945, Bensley 1910, 1951 Guyer and Claus 1932, 1934 Hirsch 1939, Parat and Painleve, 1925, Gatenby 1931, 1951, Nassonov 1923) Kedrowski (1935a, 1935b) and von Mollendorff (1938) thought that these bodies arose by a process of protoplasmic syneresis In another study (Richter, 1940), evidence was accumulated which indicated that vacuoles may arise by a process of syneresis from golgi bodies known to be comprised of both osmiophilic and osmiophobic golgi components namely the acroblast of the animal flagellate spermatid This phenomenon is evident in FIGURES 11, 12, 13 and 14 which show the progressive compaction of the particulate substance of the golgi body with the concurrent formation and final extrusion from it of a vacuole Unpublished measurements show that the volume of the original golgi structure is reduced by an amount approximately equal to the volume of the vacuole formed Guyer and Claus (1932) have made specific mention of the reduction in size of the golgi complex in certain somatic cells in parallel with the formation of vacuoles although Bowen (1929) categorically disagreed with this observation In any event the phenomenon illustrated is so far as I know, the most precise bit of evidence we have bearing on the intracellular origin of vacuoles The observation is significant not only in that it rather conclusively identifies this phenomenon as being a functional expression of the golgi complex but in that it indicates something of the basic mechanism by which it may be accomplished

In the leukocytes vacuole formation too seems somehow related to the elements of the golgi complex (*i e* as treated here) although the relations are not clear (Simpson 1930 Cunningham and Tompkins 1930, Hall 1938 Sabin 1923 Bloom 1928a b Dawson and Spark 1929 Jordan 1925 and others) Wallgren (1951 1946a 1946b) has observed vacuoles (his pale droplets) in the living lymphocytes to leave the golgi complex and enter the general cytoplasm He was unable to determine how they arise within the golgi complex Neither has this phenomenon been evident in my motion picture records of the living leukocytes although the changes in shape and size of the individual specific granules in the eosinophiles (FIGURES 5 29) and the dark droplets of the golgi system in lymphocytes as observed by Wallgren are not incompatible with those shown by the acroblast (FIGURES 11 to 14) leading to the formation of vacuoles However it is not surprising that the same or similar series of changes are not clearly evidenced by all typical golgi structures or by the specific granules if it is recalled that when the *volume* of a sphere is reduced by one half the *diameter* is reduced by only slightly more than one fourth If for instance a specific granule having a diameter of one micron were to have its volume halved its diameter would decrease by little more than two tenths of a micron—in short by an amount just barely within the limit of optical resolution This amount, even if one were looking for it would be most difficult

dicates that the droplet has a general consistency less than that of the ectoplasm of the constriction ring Similarly if the movement of the cell is such as to squeeze a droplet between the nucleus and the general ectoplasmic layer the shape of the droplet is modified on its one aspect to conform to the shape of the overlying ectoplasm but not notably modified on its nuclear aspect The later modification also is evident in circumstances in which a nuclear segment is forced between two droplets In this event neither the droplets nor nucleus are appreciably modified in shape (FIGURES 24 25) It would seem that the droplets are of about the same physical consistency as the nucleus and less than that of the ectoplasm in general It must be remembered however that the ectoplasm shows characteristic changes in its consistency from region to region when the cell is actively amoeboid This is especially true of the ectoplasm in the drag end of the cell which most frequently conforms to the shape of a droplet which might be located there if the droplet is of sufficient size (FIGURES 25 51) The physical consistency of the droplets is much less than that of the specific granules In general these observations show that the droplets are fluidic in nature and have a consistency about equal to that of the nucleus

Certain observations indicate that the droplets are delimited by a surface membrane of some type and that it has a permeability characteristic which is different from that of the general cell surface This characteristic is suggested by the fact that the lipid soluble substances benzpyrene dibenzanthracene and methylcholanthrene which are taken up from the plasma in either crystalline or molecular form by the living cell cannot be demonstrated within the droplets by U V fluorescence microscopy although they can be demonstrated at the same time in the general cytoplasm and in certain other protoplasmic organelles This is also true of the droplets elaborated by the eosinophiles and monocytes

Eosinophiles The droplets in the eosinophiles have essentially the same general set of changing intracellular relationships as those in the neutrophiles (FIGURES 5 29 54 to 69) Observations on their deformation by other cellular components indicate on the whole that they are much more fluidic and of less dense consistency than are those of the neturophiles They are easily deformed by movements of the specific granules and endoplasmic streaming A close topographic relationship exists between the droplets and the eosinophilic granules but the tendency for the latter to form relatively large compound aggregates on the droplets is usually absent The size of the droplets in this cell type ranges from that of the specific granules to relatively large ones having a diameter of several micra (FIGURES 5 29 54 to 69)

Monocytes In the monocyte the droplets while of variable size usually do not attain the large dimensions of those in the neutrophiles and eosinophiles Usually they are of about the same size or smaller than the golgi bodies with which they most frequently are associated (FIGURES 10 70 to 84) They have no characteristic relation to the mitochondria or nucleus although cell movement does effect close topographical relations between them at times My observations on the relative consistency of the droplets in this cell type are incomplete but evidence so far indicates them to be highly fluidic but more

objectives methods of approach, and in points of view, it is evident from the literature that they have been the subject of much speculation that their status as preformed structures versus *de novo* structures has not been clearly determined, that their formation and fate are generally unknown and that their general significance has not been established These are the droplets which are of primary concern in the observations being recorded here I shall refer to them descriptively as vacuolar droplets, or just merely as droplets, in order to avoid the more cumbersome terminology that has grown up about them in the past and also to avoid possible bias regarding them which has been occasioned over the years by other terms

Intracellular Relationships and Physical Characteristics of Vacuolar Droplets

Neutrophiles The droplets formed in neutrophiles have no fixed position in the cell They seem to have no intrinsic movement of their own Their disposition within the cytoplasm is dependent upon endoplasmic streaming which carries them from one region of the cell to another and into intimate relationships with each other with the nucleus with the juxtanuclear golgi substance, and with the ectoplasmic layer

A most intimate and characteristic spatial interrelationship exists between the droplets and the specific granules, the latter form constantly changing aggregates in the manner of caps on the surfaces of the droplets The associated droplets and specific granules move as one in the endoplasmic streams The length of time that a given granule maintains its position on a droplet is highly variable lasting from a fraction of a second to several minutes In every event though the granules do not remain permanently attached to the surface of the droplets

The specific granules are carried quite rapidly (in terms of seconds or fractions of seconds) in seemingly well defined but narrow and irregularly shaped streams of endoplasm which converge toward the nucleus (FIGURE 32) The movement of the specific granules from the periphery of the cytoplasm to the nucleus via the endoplasmic streams seems to be a regular and characteristic phenomenon It is frequently possible as recorded and shown by motion pictures to find granules being carried in diametrically opposite directions by such endoplasmic streams when the latter are superimposed The granules carried by endoplasmic streams converging toward the nucleus slide along in close contact with the surface of the nucleus and juxtanuclear golgi substance They finally are carried away from the nuclear region and redistributed in the general cytoplasm

Advantage was taken of amoeboid movement and its accompanying endoplasmic streaming to determine something of the general physical consistency of the droplets relative to other protoplasmic components When the droplets are lying free and unconfined in the endoplasm they always assume the shape of perfect spheres (FIGURES 15 16 17 24 25) This phenomenon indicates that they have a relatively high surface tension and a greater physical consistency than the endoplasm If in the course of amoeboid movement a droplet is forced through an ectoplasmic constriction ring its shape is progressively and characteristically modified by it (FIGURES 18, 19, 20, 21) This change in

cific granules continued to maintain a close association with the surfaces of the fusing droplets (FIGURE 36)

At 70 minutes Fusion within and between the various sized droplets continued until the cell contained only a reduced number of relatively large spherical ones (FIGURE 37) with specific granules aggregated at their surfaces

In some cells fusion of the droplets with each other may continue until only one droplet is present which may be larger than an erythrocyte (FIGURES 18 to 21)

Throughout the entire sequence of the elaboration and maturation of the droplets there is no evident structural alteration of any of the protoplasmic organelles (FIGURES 30 to 37) The cells are usually actively amoeboid throughout this period (FIGURES 30 to 37) Two constant associations are evident during the period of elaboration and maturation of the droplets The one is the relationship between the specific granules and the droplets from the time the latter are first clearly discernible the other is the intermittent association of the specific granules with the nucleus and the perinuclear golgi structure

In the eosinophiles inasmuch as the studies on this cell are incomplete it can be said of the elaboration and maturation of droplets in them only (1) that the droplets when first distinguishable are smaller than or about the size of the eosinophilic granules (2) that a close topographic association exists between the granules and the droplets from the very beginning (FIGURES 5 29 54 to 69) (3) that the association of the granules with the droplets is not permanent and (4) that visible maturation of the droplets is accompanied by the aggregation and fusion of smaller ones

On the formation of the vacuolar droplets in the monocyte my observations are incomplete but such data as I have indicates (1) that they arise in association with the typical golgi bodies of these cells (FIGURE 10) (2) that when first clearly distinguishable they have about the same size variations as the individual golgi bodies in this cell type (3) that once formed they are moved independently about in the cytoplasm and (4) that they have no permanent topographic association with any other formed element in the cell.

The extrusion of elaborated vacuolar droplets Unless cell death intervenes the droplets elaborated by eosinophiles neutrophiles and monocytes are usually extruded from the cell The phase of extrusion of the droplets into the plasma is a rapid process which varies somewhat with the size of the individual droplet and occupies on the average about one second of time I have been unable to determine any change in behavior of the droplet the cytoplasm or the specific granules which can be used as a sign to indicate that a droplet is about to be extruded Ordinarily though if a cell contains more than one droplet and one droplet is seen to be extruded the other droplets will usually be extruded quite soon after Even so one cannot tell ahead of time precisely when extrusion will occur and the flicking of an eye lid may make one miss the phenomenon The extrusion process is not polarized in these cells The droplets may be extruded from any point on the cell surface The droplets alone are extruded at least no cytoplasmic substance has been seen to be discharged along with them Extrusion occurs while the cells are actively moving and also when they are quiescent

dense than the general cytoplasmic matrix With phase, the golgi bodies are seen to be distributed as discrete dark spherical bodies of variable size (1 to 3 micra in diameter) distributed about the nucleus They have no fixed positions, however, with reference to the nucleus (FIGURE 10)

Elaboration and maturation of the vacuolar droplets In order to study critically the course of elaboration and maturation of the vacuolar droplets in the neutrophiles, single cells with no evident droplets were selected within three to five minutes after setting up the preparations and were observed continuously during the course of one to two hours At regular 10-minute intervals a short motion picture record was made of developments In the neutrophiles although there are individual variations in time from cell to cell, the essential details are the same for all The following description on the general elaboration and maturation of the vacuolar droplets in the neutrophiles is based on the record of a single representative cell which was selected within two minutes after setting up the preparation and was observed continuously for 70 minutes

At zero minutes The cell was of typical appearance with well defined nuclear structures juxtanuclear golgi structure specific granules and endoplasmic streams No vacuolar droplets were evident (FIGURE 30) The cell was actively motile

At 10 minutes The cell showed active amoeboid movement, and all the structural components were unchanged from their status at zero minutes The specific granules were seen alternately to aggregate into clumps and then to separate a movement seemingly related somehow to endoplasmic streaming There were no vacuolar droplets clearly evident at this time (FIGURE 31)

At 20 minutes Numerous spherical droplets the same size as the specific granules or slightly larger were present Such droplets when first seen and afterwards bore no special topographic relation to the nucleus They were always intimately associated with one or more specific granules Ordinarily the granules and droplets moved along as one in the endoplasmic streams (FIGURE 32)

At 30 minutes The individual vacuolar droplets were increased slightly in size Endoplasmic streaming occasioned some aggregation of the droplets to form small changing irregularly shaped clumps in which the droplets were constantly shifting positions or leaving the clumps entirely The small aggregates were distinctly unstable The specific granules remained associated constantly with the droplets (FIGURE 33)

At 40 minutes The majority of the smaller droplets were clumped and partially fused forming larger and prominent but highly irregularly shaped masses which were unstable Other droplets derived by more complete fusion of smaller ones formed discrete larger spherical ones two to three micra in diameter which were quite stable (FIGURE 34)

At 50 minutes The various sized droplets had all become fused or partially fused to form a reduced number of rather prominent droplets of irregular but stable form (FIGURE 35)

At 60 minutes The larger droplets through more complete fusion of their constituent small droplets and through fusion with other aggregates had become progressively larger and better defined as spherical droplets The spe

The cyclic nature of the phases of elaboration and extrusion of the vacuolar droplets At this time detailed studies on the cyclic nature of the whole sequence of events, including elaboration maturation and final extrusion of the vacuolar droplets have been made only on the neutrophiles

To determine whether this entire sequence was cyclic, individual neutrophiles containing fully mature droplets were selected and observed continuously until (1) the original complement of droplets was extruded, and (2) an entirely new or second complement of droplets was elaborated and finally extruded The time was recorded at all critical points during the period of observation Motion picture records in some cases were made of the critical points also It can be said at once that the entire process is cyclic and may be repeated several times The duration of the cycle varies from cell to cell The record on one neutrophile during a period of one hour and three minutes is illustrative

Preparation set up at 8 20 A M
8 37 A M a neutrophile was selected with three droplets 3 to 4 micra in diameter
End of first cycle 8 42 A M two droplets extruded
8 47 A M third droplet extruded
Beginning of second cycle 8 47 A M
9 38 A M one droplet 3 to 4 micra in diameter present
End of second cycle 9 40 A M droplet extruded

It is interesting to note in connection with this illustrative case, that the entire second cycle was begun and completed in 53 minutes and that the preceding or first cycle was completed just 27 minutes after the preparation was set up This response indicates strongly that the first cycle in this case was begun *in vivo* in the circulating blood about 26 minutes before finger puncture Jordan (1925) made a somewhat similar observation relating to this point and it is easily confirmed that no matter how rapidly a preparation is set up and placed under observation one always finds a number of cells either with fully mature droplets or droplets in various stages of elaboration and maturation In this particular instance which is quite typical the whole complement of elaborated droplets was extruded within a five minute period In some instances only part of the complement of droplets may be extruded at approximately the same time the remaining droplets may be retained for many minutes before being extruded

Depending upon the length of time that a preparation has been set up and also on the actual age of particular cells (since in any sample of whole blood the leukocytic population will necessarily be comprised of young intermediate and old cells) the number of cells which cannot extrude the elaborated droplets progressively increases (R E H Simpson 1943) The end result in such instances when cell death and lysis intervene is that the droplets are released much as they are in a holocrine type of gland cell In the younger and apparently more healthy cells the cyclic formation and final extrusion of the droplets leaves the cells with an appearance that is completely normal in every way with no discernible evidence that such cells have ever contained even one droplet

In spite of numerous motion picture records of this phenomenon little can be said concretely of the mechanism by which extrusion is accomplished It was pointed out in a previous section that the ectoplasm generally is of greater consistency than the droplets This is evident, too when the droplets pass through the ectoplasmic surface at the time of extrusion, because the droplets (especially the larger ones) are seen to be deformed much as when passing through an ordinary constriction ring (FIGURES 18 to 21) It is also evident that the droplets are under some considerable pressure from within the cell In some instances when the droplets are small they can be seen to be projected a considerable distance from the cell In every instance the droplet is moved close to the cell surface The overlying ectoplasm progressively becomes very thin and finally disappears (FIGURES 38 to 42) Simultaneously, the droplet is started on its way out (FIGURE 43) In the case of extremely large droplets one can detect a narrow equatorially situated line (undoubtedly the dense rim of ectoplasm delimiting the extrusion site) which sweeps over and behind the emerging droplet to reconstitute a continuous ectoplasmic layer at the site of extrusion This phenomenon is barely discernible in FIGURES 43 to 48, which also show the hourglass deformation of the droplet during extrusion by a neutrophile Studies are underway to analyze this phenomenon further

The extrusion phase, as just described, appears to be basically the same in all the cell types in which it has been observed to occur, namely, in the neutrophiles, eosinophiles, and monocytes The lymphocytes in my preparations rather uniformly do not elaborate these droplets and they have not, so far, been observed to extrude anything The entire series of motion picture frames covering the phase of extrusion of elaborated droplets by a representative neutrophile eosinophile and monocyte are given in FIGURES 38 to 49 54 to 69, and 70 to 85 respectively

Droplets extruded by the neutrophiles retain their identity in the plasma (FIGURES 38 to 49) In the cell illustrated, the droplet was extruded in six eighths of a second (FIGURES 43 to 48) How long they retain their identity has not yet been determined but it has been possible to keep a few droplets of neutrophilic origin under continuous observation for as long as four hours after extrusion

Droplets extruded by the eosinophiles lose their identity almost immediately upon entering the plasma (FIGURES 54 to 69) In motion picture records of the phenomenon in this cell it has been seen that an erythrocyte nearby but not in contact with the cell and yet in the line of fire is caused to jerk at the time of extrusion as if moved by a sudden eddy in the intervening plasma In the eosinophile illustrated, extrusion occurred in about five eighths of a second (FIGURES 60 to 65) as nearly as it can be made out by study of the film

Droplets extruded by the monocytes disappear immediately upon entering the plasma In the monocyte illustrated the droplet was extruded from the surface underneath and was accomplished in about seven eighths of a second (FIGURES 75 to 81)

The fact that the droplets of neutrophilic origin retain their identity in the plasma while those of monocytic and eosinophilic origin do not suggest that they are of rather different physicochemical composition

(they are necessarily of neutrophilic origin as those produced by the eosinophiles and monocytes disappear immediately upon extrusion) During the course of two hours or more however relatively large numbers are found to accumulate there It has not yet been possible to make precise quantitative measurements on the total amount of vacuolar substance contributed by the neutrophiles eosinophiles and monocytes to the plasma *in vitro* However, if it is assumed (1) that the droplets have the density of water (it seems obvious that they do not), and (2) that each neutrophile eosinophile and monocyte in 1 cc. of whole blood elaborates and extrudes one droplet 5 micra in diameter per hour it is estimated that these cells *in vitro* elaborate and add to the plasma 311 μg of vacuolar substance/hour/cc of whole blood This is a most conservative estimate in view of the observations recorded here Three hundred and eleven μg/cc is a rather striking amount of substance, especially when it is recalled that many naturally occurring and physiologically effective substances are present normally in blood in considerably less quantities

In general summary of the observations recorded in this section it seems essential to point out only that all of the data show (1) that neutrophiles eosinophiles and monocytes have the capacity to and do elaborate mature and extrude morphologically discrete and characteristic products into the plasma in a cyclic and repetitive manner (2) that these activities satisfy all of the known criteria of secretory activity generally in other glandular cell types (3) that these activities of elaboration maturation, and extrusion of formed products parallel those seen in merocrine type gland cells as long as the cells are healthy and alive (4) that when cell death intervenes the mechanism of extrusion becomes essentially that of a holocrine type of gland cell (5) that the droplets elaborated and extruded have a physicochemical composition that varies somewhat with the species of leukocyte producing them and that the droplets have a physical consistency near to that of the nucleus and greater than that of the erythrocyte (in the case of droplets of neutrophilic origin) (6) that the data suggest that the entire process occurs normally *in vivo*

VI *On the Chemical Composition of the Vacuolar Droplets*

At this time very little precise information is available on the chemical composition of the vacuolar droplets of leukocytic origin The observations recorded here indicate certain differences in physicochemical composition between the droplets of neutrophilic eosinophilic and monocytic origin It has been reported on the basis of direct and indirect evidence and irrespective of the species of leukocyte that the vacuolar droplets contain water (Renaut 1907 Dubreuil 1913 Jordan 1925 Mollendorff 1937 Heinz 1890 Dustin 1947 Kedrowski 1935a 1935b Metchnikoff 1905) and unspecified inorganic salts (Renaut 1907 Shipley 1919) The vacuolar droplets have been reported to contain an albuminous substance (Dubreuil 1913 Renaut 1907) phosphatids (Hammar cited by Dustin 1944) and proteolytic enzymes (Shipley 1919 Metchnikoff 1905) Wallgren (1946b) has reported that they probably contain a carbohydrate Kossel (1894 cited by Metchnikoff 1905) reported that the droplets contained nucleic acid and this report has been confirmed recently by direct histochemical methods (Dustin 1947 Jackson 1954) Further and

Since many studies on the leukocytes have been mediated through neutral red staining certain apparently well established effects of neutral red should be pointed out, as they have a direct bearing on the general question of the cytophysiologic status of leukocytic vacuolar droplets (1) the formation of vacuolar droplets requires metabolic energy, as shown by treatment of living cells with KCN, which is a respiratory enzyme toxin inhibiting the formation of vacuolar droplets (Kedrowski, 1935a, 1935b), (2) neutral red is known to have an enzyme precipitating effect (Koehring, 1931 Kedrowski 1932), (3) neutral red somehow disrupts or precipitates nucleic acid complexes (Jackson 1934 Dustin 1947, 1944) In view of these effects it is not surprising that neutral red staining (1) hastens cell death and (2) makes for an abnormal accumulation of vacuolar droplets within the cell as has been reported by many workers (Rosin and Biebergeil 1904 Jordan 1925, Dawson and Spark, 1929 Hall, 1931, and others) On the basis of the findings reported here it would seem that the extrusion of vacuolar droplets requires the active participation of the fibrous structural proteins of the cell cytoplasm and cell surface Since neutral red somehow disrupts cytoplasmic nucleic acid complexes and since plasma is in this category and is also a structural protein it seems permissible to suggest that the abnormal accumulation of vacuolar droplets in the leukocytes after *neutral red staining is due to a disruption of the extrusion mechanism namely,* the cytoplasmic structural proteins

Extruded droplets of neutrophilic origin have a refractive index so close to that of plasma that they are virtually indistinguishable with the ordinary light microscope With the phase microscope they are readily seen as pale droplets of variable sizes Their surfaces are usually clean and smooth although hematoconia and chylomicra frequently dance into contact with and about them Each droplet seems to be delimited by a surface membrane Ordinarily they round up immediately upon entrance into the plasma (FIGURES 53 48 49) Sometimes they do not and in this case they remain aspheric and show much surface activity with consequent rapid changes in shape (FIGURE 53) The latter changes are most similar to those seen within the cell in connection with the early phases of maturation of the droplets when in the process of fusion This phenomenon suggests that the extracellular aspheric droplets are instances wherein extrusion occurred before the fusion of the smaller droplets during maturation was completed This suggestion is supported by cinematographic records of instances in which droplets which appeared to be perfectly fused while within the cell were aspheric after extrusion (FIGURES 51 52)

On the basis of deformational evidence, it can be demonstrated that the extruded droplets of neutrophilic origin have a general consistency greater than that of the mature erythrocyte *This characteristic is clearly evidenced by* selected frames from a motion picture sequence showing the extrusion of a droplet which later on when crowded by the body of its parent cell against an erythrocyte deforms the latter without itself being deformed in any way (FIGURES 46 to 50)

When one surveys the entire field of a preparation within a few minutes after it is made only an occasional droplet or no droplets can be found in the plasma

cytes of such exudates as well as those of normal buffy coat, of media modified *in vitro* by buffy coat leukocytes and of stored leukocytes contained a substance which promoted cell growth and another which hemolyzed foreign red cells These workers interpreted their findings as evidence that the leukocytes have a secretory function Crand (1949) has reported that fowl leukocytes grown in suspensions of *Staphylococcus aureus* have an antibiotic effect Mammalian leukocytes did not exhibit this effect The studies of Sabin (1939) also have a significant bearing on the status of the leukocytic vacuolar droplets Using a synthetic red-dye protein antigen Sabin found that it was taken up *in vivo* by neutrophiles monocytes and macrophages and localized intracellularly in vacuolar droplets She noted that the appearance of antibody in the serum coincided with the time when the dye protein antigen was no longer visible in the cell and with the shedding of the ectoplasm of the monocytes and phagocytes She visualized the release of antibody from the cells as mediated by the shedding of small globules of ectoplasm It is possible that the latter are extruded vacuolar droplets such as I have reported here

In summary of this section it can be said that the majority of workers and the bulk of the evidence direct and indirect support the view that leukocytes have a secretory (and synthesizing) capacity, and that the vacuolar droplets are normal morphologic expressions of this capacity

General Summary

On the basis of the observations recorded here and of their close correlation with the findings of many others it seems necessary to conclude that the neutrophiles eosinophiles and monocytes have a typical (merocrinelike) secretory capacity Although the vacuolar products of this activity most characteristically are associated with the elements of the golgi complex intrinsic movements of the cell constantly effect changing spatial relationships between them and other protoplasmic organelles such as the mitochondria nucleus golgi elements ectoplasm and general cytoplasmic matrix None of the observations made in these studies on the living cells and recorded cinematographically can be taken as evidence precluding the possibility that mitochondria nuclei nucleoli or general cytoplasmic matrix may participate in the secretory phenomena described here The evidences seem rather to enhance the probability that all of these special protoplasmic components are active in some fashion in one or another of the facets of the secretory process

Acknowledgments

I wish to express publicly my sincere appreciation and thanks to Georgia A Stathos Robert E Coalson Neil Woodward Jr Paul B McCay Ramon Mongulia and I H F Timmons for their untiring cooperation and technical assistance

Bibliography

ALEKSANDROWICZ J J BLICHARSKI & I FELTYNOWSKI 1952 Przyczynek do morfologii granulocytow w swietle badan elektronskopowych Doniesienie tymczasowe Polski Tygodnik Lekarski 7(51-52) 1765-1766

more precise qualitative and quantitative studies on the chemistry of these droplets in the various species of leukocytes are sorely needed

VII On the General Status of the Vacuolar Droplets

It was suggested in a preceding section that the vacuolar droplets have enjoyed a rather unsettled status over the years To some workers (Cesaris-Demel 1909, Jackson, 1934, Bessis, 1949) the presence of vacuolar droplets in the leukocytes was a sign of cellular degeneration, and to these investigators the droplets were accordingly known as degeneration vacuoles The evidence for this point of view so far as the leukocytes is concerned principally stems from the correlation made by Cesaris Demel (1909) that, under many conditions including infections, the circulating leucocytes, as well as those in purulent exudates pleural exudates *etc* contained vacuolar droplets On the other hand, Marini (1900 cited by Cesaris Demel, 1909), in experimental infections with pneumococci and in cases of fatal pneumonia, made the same observation on the presence of vacuolar droplets in the leukocytes but Marini did not interpret their presence as a degenerative change, but rather as a sign of a different kind of function of these cells "d h derjenigen Funktion derselben, durch welche die zur Hervorbringung der Immunitat dienenden Produkte abgesondert werden (Cesaris-Demel 1909, S 8) I am not aware of any compelling and incontrovertible evidence supporting the belief that vacuolar droplets, *per se* are signs of cellular degeneration and that they have no further significance

While the particular significance which has been attached to leukocytic vacuolar droplets varies from one group of workers to another the vast majority do not consider them to be degenerative manifestations Simpson (1921) and Sabin (1923, 1939) considered them to be associated with intracellular segregation (Renaut 1907), detoxification and excretory processes Jordan (1925) considered them normal cytoplasmic structures and inclined to view them in some sort of excretory role Key (1928) considered them to be a normal cellular component and, when their numbers increased in the cells viewed them as a normal response to stimulation and not degenerative manifestations Shipley (1919) and Opie (1922) considered them a normal cellular component and viewed them as sites of enzymatic digestion (Metchnikoff 1905) Mollendorff (1937) considered them to be associated with the regulation of protoplasmic viscosity and others have shown that their formation may be prevented or augmented by hypertonic and hypotonic media respectively (Heinz 1890 Kedrowski 1935a) The majority of workers have considered them to be normal constituents and to be specific secretory products and/or associated with synthesizing activities (Kossell 1894 cited by Metchnikoff 1905 Marini 1900 cited by Cesaris-Demel 1909 Renaut 1907 Dubreuil 1913 Goldmann 1912 Ehrich 1934 Nagel 1929 cited by Ehrich 1934 Kedrowski 1932 1935a, 1935b Dustin 1947 1944 1943 Wallgren 1951 1946a 1946b Richter 1954)

Since it is well-established that the leukocytes of pleural and peritoneal exudates and of inflammatory sites typically contain vacuolar droplets it seems significant to the status of the latter that Carrel (1922 1924) Carrel and Ebeling (1922a, 1922b) and Kruschov (1945) found that the extracts of the leuko-

CRAMPTON C F & F HAUROWITZ 1950 Intracellular distribution in rabbit liver of injected antigens labelled with I[131] Science 112 300-30
CUNNINGHAM R F SABIN & C DOAN 1925 The development of leucocytes lymphocytes and monocytes from a specific stem cell in adult tissue Contrib Embryol Carnegie Inst Wash 16 227-276
CUNNINGHAM R & E H TOMPKINS 1930 The supravital staining of normal human blood cells Folia Haematol 42 257-270
DALTON A J 1953 Electron microscopy of tissue sections Inter Rev Cytol 2 403-417
D'ANGELO S A 1941 An analysis of the morphology of the pituitary and thyroid glands in Amphibian metamorphosis Am J Anat 69 407-437
DAWSON A B 1942 Some morphological aspects of the secretion process Federation Proc 1 233-240
DAWSON A B & C SPARK 1929 The reaction of the segregation apparatus of the thrombocytes and leucocytes of the blood of *Necturus* to neutral red and to prolonged exposure to osmic acid Anat Record 41 335 357
DEINEKA D 1912 Der Netzapparat von Golgi in einigen Epithel und Bindgewebszellen während der Ruhe und während der Teilung derselben Anat Anz 41 289-309
DE ROBERTIS E 1940 The cytology of the parathyroid gland of rats injected with parathyroid extract Anat Record 78 473-497
DISTOMBE G 1946 Extrusion of nucleic acid from the nuclei of human granulocytes Nature 157 370-371
DOUGHERTY T F J CHASE & A WHITE 1944 The demonstration of antibodies in lymphocytes Proc Soc Exptl Biol Med 57 295-298
DOUGHERTY T F J CHASE & A WHITE 1945 Pituitary adrenal cortical control of antibody release from lymphocytes An explanation of the anamnestic response Proc Soc Exptl Biol Med 58 135-141
DOWNEY H 1913 The origin of blood platelets Cytoplasmic shedding Folia Haematol 16 25-58
DOWNEY H 1928 Special Cytology 1 3 0-399 E V Cowdry Ed Hoeber New York N Y
DOWNEY H 1930 Textbook of Pathology Bell Ed Lea & Febiger Philadelphia Pa
DUBREUIL G 1913 Le chondriome et le dispositif de l'activité sécrétoire aux différents stades du développement des éléments cellulaires de la lignée connective descendants du lymphocyte (globules blanc mononucléés de la lymphe et du sang cellules connectives cartilagineuse et osseuses) Arch d anat microscop 15 53
DUSTIN P 1941 Contribution à l'étude de la coloration vitale granulaire par les colorants basiques Production expérimentale de granules basophiles dans les erythrocytes et les tissues des amphibiens Acad roy Belg 27 612-675
DUSTIN P 1944 Contribution à l'étude hi topl ysiologique et histochimique des globules rouges des Vertébrés Arch biol 55 285-392
DUSTIN P 1947 Ribonucleic acid and the vital staining of cytoplasmic vacuoles in animal cells Symp Soc Exptl Biol No 1 114-126 Cambridge Univ Press Cambridge England
EHRICH W 1934 Die Leukozyten und ihre Entstehung Ergeb allg Path u path Anat 29 1-144
ENGELBERT V E 1953 Cells of the thymus and their release of cytoplasmic portions *in vivo* Can J Zool 31 106-111
ERÄNKÖ O 1951 The structure of granulocytes Duodecim 67 239-248
ESTABLE C 1931 Observations sur l appareil de Golgi dans les cellules du sang et des organes hématopoiétiques Compt rend soc biol. 106 841-844
FAGRAEUS A 1948 Antibody production in relation to the development of plasma cells Acta Med Scand Suppl 204 130 1-122
GATENBY J B 1931 The prozymogen granules (vacuome) of R R Bensley in the *Pseudo ... tion* pancreas and the modern neutral red cytology Am J Anat 48 421-477
GATENBY J B 1951 The golgi apparatus of liver and nerve cells Nature 167 185-186
GRAND C G 1949 Antibiotic effects of fowl leucocytes *in vitro* Anat Record 10 488
GREPP R 1954 Histology 26-28 Blakiston New York N Y
GRESSON R A R 1952 The golgi substance La Cellule 54 398-403
GURBER A 1890 Untersuchungen über die physiologischen Wirkungen des Serpetidine und verwandter Körper und Beziehungen zu ihrer chemischen Constitution Arch Phys ol Jahrgang 1890 401-477
GUYER M F & P E CLAUS 1932 Cell changes in the anterior lobe of the pituitary following cancer transplantation in rats Anat Record 52 225-239
GUYER M F & P E CLAUS 1934 The golgi apparatus in relation to vacuolation in the

ARRIGONI C 1908 Über die Metamorphose des Kernes der menschlichen Erythroblasten und uber die Natur der chromatophilen Substanz der Erythrozyten Folia Haematol 6 444–470
BAKER B L 1942 A study of the parathyroid glands of the normal and hypophysectomized monkey (*Macaca mulatta*) Anat Record 83 47–73
BAKER J R 1944–45 The structure and chemical composition of the golgi element Quart J Microscop Sci 85 1–72
BAKER J R 1950 Morphology and fine structure of organisms Nature 165 585–586
BAKER J R 1951 The Golgi substance Nature 168 1089–1090
BENNETT H S 1940 The life history and secretion of the cells of the adrenal cortex of the cat Am J Anat 67 151–227
BENSLEY R R 1910 On the nature of the canalicular apparatus of animal cells Biol. Bull 19 179
BENSLEY R R 1951 Facts versus artefacts in cytology The golgi apparatus Exptl Cell Research 2 1–9
VON BERGEN F 1904 Zur Kenntnis gewisser Strukturbilder (Netzapparate Saftkanälchen Trophospongien) im Protoplasma verschiedener Zellenarten Arch mikroskop Anat Entwicklungsmech 64 498–574
BESSIS M 1949a Études sur les cellules sanguines au microscope a contraste de phase et par la méthode de l ombrage Rév Hemat 4 294–349
BESSIS M 1949b Études sur les cellules des leucemis et des myélomes au microscope a contraste de phase et par la méthode de l ombrage Rév Hemat 4 364–394
BLOOM M L & G B WISLOCKI 1950 The localization of lipids in human blood and bone marrow cells Blood 5 79–88
BLOOM W 1928a The relationships between lymphocytes monocytes and plasma cells Folia Haematol 37 63–69
BLOOM W 1928b The origin and nature of the monocyte Folia Haematol 37 1–62
BLOOM W 1928c Mammalian lymph in tissue culture From lymphocyte to fibroblast Arch exptl Zellforsch Gewebezucht 5 269–307
BLOOM W 1938 Handbook of Haematology 1 375–435 Hoeber New York N Y
BOWEN R H 1924 Possible relation between golgi apparatus and secretory products Am J Anat 33 197–217
BOWEN R H 1925 Further notes on the acrosome of the animal sperm The homologies of non flagellate perms Anat Record 31 201–232
BOWEN R H 1926 The golgi apparatus—its structure and functional significance Anat Record 32 151–193
BOWEN R H 1927 Golgi apparatus and vacuome Anat Record 35 309–335
BOWEN R H 1929a The cytology of glandular secretion Quart Rev Biol 4 299–324
BOWEN R H 1929b Cytology of glandular secretion Quart Rev Biol 4 484–519
BRUGSCH T & V SCHILLING 1908 Die Kernform der Neutrophilen Leukocyten beim Menschen Folia Haematol 6 327–336
BUNTING C H 1928 Special Cytology 1 403–424 E V Cowdry Ed Hoeber New York N Y
BUNTING C H 1928 Special Cytology 1 161–177 E V Cowdry Ed Hoeber New York N Y
BURNET F M & F FENNER 1949 The Production of Antibodies 1–133 2nd ed Macmillan Melbourne Australia
BUTTERFIELD E E A HEINEKE & E MEYER 1909 Über das Vorkommen der altmannschen granulationen in den weissen Blutzellen Folia Haematol 8 325–333
CARREL, A 1922 Growth promoting function of leucocytes J Exptl Med 36 385 391
CARREL A 1924 Leucocytic trephones J Am Med Assoc 82 255–258
CARREL A & A H EBELING 1922a Pure cultures of large mononuclear leucocytes J Exptl Med 36 365–377
CARREL A & A H EBELING 1922b Leucocytic secretions J Exptl Med 36 645–659
CASPERSSON T O 1947 The relations between nucleic acid and protein synthesis Symp Soc Exptl Biol 1 127–151
CASPERSSON T O 1950 Cell Growth and Function 11–185 Norton New York N Y
CESARIS DEMEL A 1909 Über die morphologische Struktur und die morphologischen und chromatischen Veränderungen der Leukozyten Virchow s Arch path Anat 87 1–92
COWDRY E V 1914 The vital staining of mitochondria with Janus green and diethyl safranin in human blood cells Intern Monatsch Anat u Physiol 31 267
COWDRY E V 1921 The reticular material of developing blood cells J Exptl Med 33 1–12

mente in der embryonalen Entwicklung und in postfötalen Leben der Saugetiere Folia Haematol 8 125–134
MAXIMOW A 1927 Über das Mesothel (Deckzellen der serosen Haute) und die Zellen der serosen Exudate Untersuchungen an entzundetem Gewebe und an Gewebskulturen Arch exptl Zellforsch Gewebezücht 4 1–42
MAXIMOW A 1928a Special Cytology 1 319–367 E V Cowdry Ed Hoeber New York N Y
MAXIMOW A 1928b Cultures of blood leucocytes From lymphocyte and monocyte to connective tissue Arch exptl Zellforsch Gewebezücht 5 169
MAXIMOW A 1928c Special Cytology 1 319 367 E V Cowdry Ed Hoeber New York N Y
MAXIMOW A & W BLOOM 1930 A Textbook of Histology Saunders Philadelphia Pa
METCHNIKOFF E 1905 Immunity in Infective Diseases 1 91 Transl by F G Binnie Cambridge Univ Press 1907 Cambridge England
MEVES F 1896 Über die Entwickelung der männlicher Geschlechtszellen von *Salamandra* Arch mikroscop Anat Entwicklungsmech 48 1 83
MÖLLENDORFF W VON 1920 Vitale Farbungen an tierischen Zellen Grundlagen Ergebnisse und Ziele biologischer Farbstoffversuche Ergeb Physiol 18 141–306
MÖLLENDORFF W VON 1937 Beitrage zum Problem der Zellenviskositat Arch exptl Zellforsch Gewebezücht 19 263–275
MÖLLENDORFF W VON 1938 Experimentelle Vakuolenbildung in Fibrozyten der Gewebeskultur und deren Farbung durch Neutralrot Protoplasma 30 475
NAEGELI O 1931 Blutkrankheiten und Blut Diagnostik 5th ed Springer Berlin Germany
NASSONOV D 1923 Das golgische Binnennetz und seine Beziehungen zu der Sekretion Untersuchungen über einige Amphibiendrüsen Arch mikroscop Anat Entwicklungsmech 97 136–186
NOEL, R & H PIGEAUD 1930a Sur la nature mitochondriales des granulations eosinophiles des leucocytes chez le foetus humain Compt rend soc biol 104 09–210
NOEL R & H PIGEAUD 1930b Mitochondria of hematopoietic elements in liver of human fetus Bull histol appl physiol et path et tech microscop 7 167–166
OPIE E L 1922 Intracellular digestion the enzymes and anti enzymes concerned Physiol Revs 2 552–585
PALADE G & A CLAUDE 1949a The nature of the golgi apparatus I Parallelism between intercellular myelin figures and golgi apparatus in somatic cells J Morphol 85 35–69
PALADE G & A CLAUDE 1949b Nature of the golgi apparatus II Identification of the golgi apparatus with a complex of myelin figures J Morphol 85 71–111
PANTEL J & R DE SINETY 1906 Les cellules de la lignée male chez l *Notonecta glauca* La Cellule 23 89–298
PARAT M & J PAINLEVÉ 1925 Rôle du vacuome (appareil de Golgi) et du chondriome dans la formation des grains de sécretion Compt rend soc biol 92 65–67
PARRISIUS W & W SCHLOPSNIES 1927 Über die Absterbevorgärge on den weissen Blutkörperchen *in vitro* Folia Haematol 34 90–102
PAYNE F 1927 Some cytoplasmic structures in the male germ cells of *G lastocoris oculatus* J Morphol 43 299–345
PAYNE F 1940 Signet ring or castration cells in the chick Anat Record 76 29 37
PIERCE M 1932 Cultures of leukemic blood leucocytes Arch Pathol 14 295–322
RENAUT J 1883 Traité d histologie pratique Vol 1 Pt 1 79–94 Paris France
RENAUT J 1907 Les cellules connectives rhagocrine Arch Anat Microscop 9 495 605
RHOADS C P AND F PARKER 1928 Observations on incubated normal blood Am J Path 4 271 84
RICHARDSON K C 1934 *Golgi apparatus in normal and degenerate cells in vitro* Arch exptl Zellforsch Gewebezücht 16 100–115
RICHTER K M 1939 Cytoplasmic inclusions in the male germ cells of *Notonecta undulata* Sav Biologist 19 140–111
RICHTER K M 1940 A study of the cytoplasmic inclusions in the male germinal cells of several species of *Notonecta* with special reference to the golgi system J Morphol 67 489–521
RICHTER K M 1942 An experimental study of the cytology of human peripheral blood neutrophiles and lymphocytes J Morphol 71 53–75
RICHTER K M 1951 The reaction of peripheral blood leucocytes in vitro to certain carcinogen crystals Anat Record 109 82

basophiles of the anterior pituitary of castrate and of cancerous rats Anat Record 61 57–69
HALL, B 1931 A critical review of the hematological literature dealing with the results of the supravital staining method Folia Haematol 43 206–234
HALL B 1938 Handbook of Haematology 1 641–698 Hoeber New York N Y
HARTLEY G 1940 The local formation of antivaccinial antibodies by the skin J Infectious Diseases 66 44–52
HAUROWITZ J 1953 The Nature and Significance of the Antibody Response 3–12 Columbia Univ Press New York N Y
HEINZ R 1890 Arbeiten aus dem pharmakologischen Institut der Universität Breslau I Die Wirkung concentrirter Salzlosungen Arch path Anat 4 100–124
HERTWIG G 1929 Handbuch der mickroskopischen Anatomie des Menschen 1 255–327 Springer Berlin Germany
HIBBARD, H 1945 Current status of our knowledge of the golgi apparatus in the animal cell Quart Rev Biol 20 1–19
HIRSCH G C 1939 Form und Stoffwechsel der Golgi Korper Protoplas Monogr 18 1–394
HOFFMAN G T & A ROTTINO 1950 Phase microscopy studies of Hodgkin s disease Lymph nodes in relation to histogenesis of the Sternberg Reed cell Blood 5 74–78
ITO T, T TAKAHASHI & Y MITZUTANI 1938 Golgi apparatus of plasma cell with special consideration of nature of its light area Okajimas Folia anat japon 16 303–314
JACKSON J F 1954 The cytology of the degenerating leukocyte J Lab Clin Med 43 227–234
JORDAN H E 1925 A study of the blood of the leopard frog by method of supravital staining combined with injection of India ink into dorsal lymph sac With special reference to the genetic relationships among leucocytes Am J Anat 35 105–132
KEDROWSKI B 1932 Uber die Natur des Vakuomes Z Zellforsch u mikroskop Anat 16 731–760
KEDROWSKI B 1934 Untersuchungen über die Kondensatoren für Basische Farbstoffe III Mitteilung Die Rolle der Euverssablauprodukte bei der Bildung der Kondensatoren (Farbstoffgranula) und bei der Farbspeicherung Z Zellforsch u mikroskop Anat 22 399–410
KEDROWSKI B 1935a Untersuchungen über die Kondensatoren für basische Farbstoffe Mitteilung I Genese der Farbstoff granula in roten Blutkörperchen des Frosches
KEDROWSKI B 1935b Untersuchungen über die Kondensatoren für basische Farbstoffe Mitteilung II Wirkung von KCN und Licht auf die Vitalfarbung der Froscherythrozyten Protoplasma 22 607–615
KEY J A 1928 Cytology of the synovial fluid of normal joints Anat Record 40 193–211
KIRKMAN H & A E SEVERINGHAUS 1938a A review of the golgi apparatus Part I Anat Record 70 413–431
KIRKMAN H & A E SEVERINGHAUS 1938b A review of the golgi apparatus Part II Anat Record 70 557–573
KIRKMAN H & A E SEVERINGHAUS 1938c A review of the golgi apparatus Part III Anat Record 71 79–103
KOEHRING V 1931 Thermal relationships in neutral red reaction J Morphol 52 165–194
KOLLER P C 1947 The experimental modification of nucleic acid systems in the cell Soc Exptl Biol Med Symp I Nucleic Acids 270–290
KRUSCHOV G K 1945 The role of the leukocytes of the blood in reparative processes in the tissues Moscow Pubs Acad Sci U S S R
LA COUR L F 1944 Mitosis and cell differentiation in the blood Proc Roy Soc Edinburgh 62 73–85
LAVDOVSKY M 1877 Zur feineren Anatomie und Physiologie der Speicheldrusen Arch mikroscop Anat Entwicklungsmech 13 ?
LAWRENCE J S & H TODD 1931 Variations in the characteristics of lymphocytes in supravital preparations Folia Haematol 44 318–331
LETTRÉ H & M ALBRECHT 1943 Zur Wirkung von Aminen auf *in vitro* gezuchtete Zellen Z physiol Chem 279 206–208
LEWIS W H & M I RUBIN 1932 Changes in the neutrophiles and monocytes in cultures of the buffy coat of human blood in serum Anat Record 53 249–254
MARINI G 1900 Modification de structure des leucocytes à noyau polymorph etc Compt rend du 13 Cong de Med Paris (see Cesaris-Demel 1909)
MAXIMOW A 1909 Der Lymphozyt als gemeinsame Stamzelle der verschiedenen Blutele

WORLEY L G 1944b Studies of the vitally stained golgi apparatus III The methylene blue technique and some of its implications J Morphol 75 261 290
WORLEY L G 1946 The golgi apparatus—An interpretation of its structure and significance Ann N Y Acad Sci 47 1 56
WORLEY L G 1951 Recovery of the golgi apparatus from homogenates of normal mammalian liver Exptl Cell Research 2 684-685

PLATES

E planation f Plat

All ll t t are tou h d photom gr ph o op of sel t d l am f m neph tom mgr ph F gur 6 t 9 were t k w th a 35 mm Le m th d rk-cont t m d m ph se q pme t w h l immers b t e l t 1 m gn fi t th film w X323 d larged pr t g t X1232 F ra s 11 t 34 h d 1 m gn fi t on t th film f X323 d re l ged p t g t pp m t l X2125
Fig 2 3 27 d 28 w re t k with 35-mm Le m a omb t w h L fluo sc m o-scope Th m gn cation t film w X323 a d w re la g d p t g t X825
All othe figures re p f sele ted f m from ph tom g ph t k w h 16 mm B l d C ne Spe l ame perat g t h t pe d f 8 fram pe se d omb on th h gh-dry d l mm d rk-contr t m d m ph se qu pm t F s 1 5 10 to 26 d 29 t 37 h d t l m gn p t o t film f X291 d we la ged in p t g t X1973 F r s 15 t 21 w re m gn h d X329 t film d l ged p t g t X877 F r 38 t 53 54 t 69 d 0 t 85 w re m g fi d X291 t th film d la ged pri t g t X1113

RICHTER K M 1952a U V fluorescence studies on the solubility and intracellular localization of certain carcinogens in human blood cells in vitro and in cells of embryonic chick heart cultures Anat Record 112 78–79
RICHTER K M 1952b A cinematographic record of the reaction pattern of human peripheral blood leucocytes in vitro to crystalline methylcholanthrene Anat Record 112 172–173
RICHTER K M 1954 A cinematographic record on human peripheral blood leucocytes in vitro bearing on the question Do leucocytes have a typical (glandular) secretory capacity? Anat Record 118 442–443
ROSIN H & E BIEBERGEIL 1904 Das Verhalten der Leukocyten bei der vitalen Blutfarben Arch path Anat Physiol Virchows 178 478–504
SABIN F R 1923 Studies of living human blood cells Bull Johns Hopkins Hosp 34 277–288
SABIN F R 1938 Cellular reaction to tuberculo-proteins compared with the reaction to tuberculo-lipids J Exptl Med 68 837–852
SABIN F R 1939 Cellular reactions to a dye protein with a concept of the mechanism of antibody formation J Exptl Med 70 67–82
SABIN F R C R AUSTRIAN R S CUNNINGHAM & C A DOAN 1924 Studies on the maturation of myeloblasts into myelocytes and on amitotic cell division in the peripheral blood in subacute myeloblastic leucemia J Exptl Med 40 845–871
SAGUCHI S 1920 Studies on the glandular cells of the frog pancreas Am J Anat 26 347–421
SCHILLING V 1929 The Blood Picture 7th & 8th eds Trnsltd by R B H Gradwohl Mosby St Louis Mo
SCHRIDDE H 1907 Weitere Beobachtung uber die lymphozytaren Zellen des Menschens Folia Haematol 4 606–607
SCHRIDDE H 1907a Ziegler s Beiträge path Anat u allgem Path 41 (see Thorell 1944)
SCHULTZE M 1865 Ein heizbarer Objecttisch und seine Verwendug bei Untersuchungen des Blutes Arch mikroscop Anat Entwicklungsmech 1 14–42
SCHULTZE F E 1867 Epithel und Drusenzellen Arch mikroskop Anat 3 145–197
SCHWIND J L 1950 The supravital method in the study of the cytology of blood and marrow cells Blood 5 597–672
SEVERINGHAUS A E 1933 A cytological study of the anterior pituitary of the rat with special reference to the golgi apparatus and to cell relationship Anat Record 57 149–175
SHIPLEY P G 1919 The physiological significance of the reaction of tissue cells to vital benzidene dyes Am J Physiol 49 284–301
SIMPSON M E 1921 On the reaction of living blood cells to dyes Anat Record 21 82
SIMPSON M E 1930 Literature on vital and supravital staining of blood blood forming and connective tissues Folia Haematol 42 247–253
SIMPSON M E & J M DEMING 1927 The identification of myeloblasts with vital stains Folia Haematol 34 103–124
SIMPSON R E H 1943 Criteria of life and death in human pus cells Brit J Exptl Path 24 218–223
SOSA J M 1949 The morphological chemical and physico-chemical significance of the golgi apparatus Exptl Cell Research (Suppl 1) 402–413
THORELL B 1944 Behavior of the nucleolar apparatus during growth and differentiation of the normal blood cells in the adult stage Acta Med Scand 117 334–375
TOMPKINS E H & R S CUNNINGHAM 1938 Handbook of Hematology 1 585–640 Hoeber New York N Y
UHLENHUTH E 1934 The golgi apparatus in the thyroid gland of amphibians in its relation to excretion polarity Quart J Microscop Sci 76 615–646
WALLGREN I 1946a On the pale granular substance of the cell and the structure of the living mass Report I The cytoplasm Acta Path Microbiol Scand 23 415–435
WALLGREN I 1946b On the pale granular substance of the cell and the structure of the living mass Report III Golgi s apparatus Acta Path Microbiol Scand 23 449–456
WALLGREN I 1951 Observations on the golgi apparatus in living plasma cells and in erythroblasts with dark ground illumination Exptl Cell Research 2 10–19
WILSON C P & R S CUNNINGHAM 1929 A consideration of the supra vital method of studying blood in cases of mononuclear cell response Folia Haematol 38 13–27
WORLEY L G 1944a Studies of the vitally stained golgi apparatus II Yolk formation and pigment concentration with mussel *Mytilus californianus* Conrad J Morphol 75 77–101

WORLEY L. G. 1944b Studies of the vitally stained golgi apparatus III The methylene blue technique and some of its implications J Morphol 75 261–290
WORLEY L. G. 1946 The golgi apparatus—An interpretation of its structure and significance Ann N Y Acad Sci 47 1 56
WORLEY L. G. 1951 Recovery of the golgi apparatus from homogenates of normal mammalian liver Exptl Cell Research 2 684–687

PLATES

Explanation of Plates

All [illegible] photom[illegible] select[ed] fram[es] [illegible] photom[illegible]. Figures 6 to 9 [illegible] with [a] 35 mm Le[ica] camera with d[ark]-contrast [illegible] phase eq[uipment] [illegible] immers[ion] object[ive] [illegible] film was ×323 [illegible] [enlar]ged [illegible] to ×123 [illegible] 11 to 14 [illegible] film [illegible] ×323 [illegible] [enlar]ged [illegible] ×2125.

Figures 23 27 [and] 28 [illegible] with [a] 35 mm Le[ica] [illegible] micro-scope. Th[e] m[a]gn[ification] at [illegible] film w[as] ×323 [illegible] ×825.

All other figures are [illegible] select[ed] [illegible] photomic[rograph] [illegible] with 16 mm B[ell] [and] [Howell] C[ine] Spe[cial] [camera] [illegible] [with] high-dry [and] oil imme[rsion] dark-contrast [illegible] Figures 5 10 22 to 5[illegible] [an]d 29 to 3[illegible] [illegible] film [illegible] ×291 [illegible] [en]larged [illegible] ×3973 [illegible] 15 [illegible] 21 [illegible] ×179 [illegible] [en]larged [illegible] ×877 [illegible] 38 to 53 54 [illegible] 69 [an]d 70 to 85 were [illegible] ×271 [illegible] the film [an]d [enlar]ged [illegible] ×1113







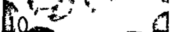










P I

FIGU 1 L g m ll l mph yt f m h g l d t l d ph l d pl g lg body ppe l ft ll l zed w th ph se

F g 2 L g h m m ll lymphocyt t d w th be p v w d th d ry l ght h g l d m ll d k ph l j t le g lg b l

F g 3 Sm ll U 2 b t l d w th th U V fl sc m pe h g th pe l t ll la l l 1 f th fl sc g be ry w th th g lg bod

F g 4 Sm ll lymphocyt f m h l Ch mpy l l ed by m mj g t l w g th th l l pl g lg body C mp w th l 2 d l

F g A l ge p h l f m l d th p l se h w g l h j t l d p l h t f th p h l g l l th l l t m ll l d p l t

F g 6 A m t pe p h l bl od t p h l f m fi d Cl mpy l l lt m mp g t h w g th po t m ge f th osm p l pe l g lg tru t

FIGU 7 A m t pe ph l blood t p h l f m wh h h d g l fl f d Ch mpy d l d w th ff h l l ght g l w g th m m p h l l th l g t mag f th pe l m ph l g lg t t

F g 8 A m t re t p h l f m wh h h d g l co fl f d Cl mpy l t d w th d f h d l ght gr h w g three t t t l l l

FIGU 9 A m t t p h l f m wh h h d g t fl wh h w f l Cl d mp regn t d w th osm d how g th po t bl k l m g f th pe l osmi h l g lgi ture

FIGU 10 A l g m ocyt f m l zed with phase how g n l ssoc t d w th da k g lg bod d d k fl th yt









PLA E 2

F GUR 11 to 14 Spe m t l f m th m y t f N t t f d B d d t d w th h m toxyl h w g t t l t ge th p gr (11 th gh 14 pe t ly) f rm t l t f l l by pro f y f m th d k l g r h l l t l g l b dy (bla t) F t 11 h w th g lg body bef th p b g s 14 h w th t t d g lg bod ft th p oc mpl t d Comp th ze d l m f th t m l g lg body u g 14 w th th se f th g lg b dy 11

P 3

FIGUR S 15 t 17 Sel t d f m f m ph se t t m t ; t seq l g t ph l f m n how ng th ge l ppe d h g g l t h p f l d pl t wh h ppe w th t cyt pla m m lky wh t ph l tru t

FI URES 18 t 21 Sele t d f am f m ph se t t m t ; t seq l g h m t o-ph l o ta g ly la ge cu la d pl t Th seq h w th prog (F 18 t 21 pe t ly) d f rmat f th d pl t by th topl m i y t f l th gh st t g

FIGU ES 22 to 23 Sel t d fram (14 f m 19 se d pa t th g l seq) f m m t p t re sequ c l g h ma pe ph l blood t ph l wh h h d l go l ff v t (ompa ed w th F GUR S 7 t 9) Th m m rph l ta se l d k t t t l le th pe cle r g lgi tru t re ppe rr gula l h ped le re bout th le N t the d ff ences in the configur t f th s struct re in FIGURES 22 d 23 wh h occu red d r g 19 se d

P TE 4

FIGU 24 to 25 Sele t d f am f om phase co t t m t p t re seq n t t ph l f m ta g cuol d pl ts Th ppe ll (g 24) h w le segm t be g f ced (URE 2) bet ee tw relat l la g d pl t ll t t g that th d plet h phy ical tency bo t qual t th t f th l us Th low ll ll t te re 4 d 25 re t ly that th uol d pl ts ha phys l t cy ffi t t d f rm th t pl m lay f th d g f f th cell

FIGU 26 A sel t d f am f om ph se t t m io p t seq ce hum eut ph le (ppe gh ll) tain g se l rously s ze d pl t w th pl k ggr g t f pe in gra le the surfaces.

FIGUR 27 A l g h m t ph l re t d with be zpy d ewed with dinary l ght h wing th le segm ts d ph l lump f pe fi gran le

FIGUR 28 Th sam ll GUR 7 b t u liz d w th th L V fl sce m oscope howing th pecial tr ll la loc l zati f th fl scing be zpyre th pe fi granule

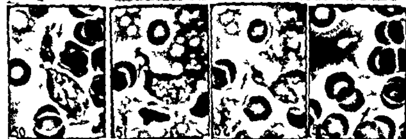
30 31 32 33 34 36 35 37

[illegible]









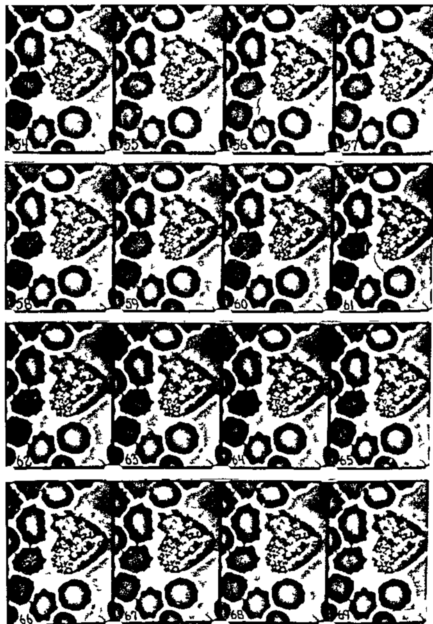
P   e 6

F   38 to 49 C se t f m (h f m g l se o d) f m ph se t a t m t p t seque t t h l h g t d g d plet t th pl m w 38 t t d pl t w h be m d d E ru on beg p m tely 43 nd m t d 10, 44 pl m t ly f se te the d pl t ta t d t ty th pl m

F 50 A f d f m bseq t f t t how g th d pl t wh h w t d d b g g d by re ll th ocyt h h t d f m

F 51 52 Sel t d f am f m ph se t t m t p t que g t how g d pl 51 m k d by w) w h ft t m d a h l n th plasm 52 m k d by w)

F 53 A sel ted f am f om ph se t t m t p t re n h g typ l h d pl t d ph d pl t f pl g t th pl m The d plets re m k d by w



P 7

FIGURES 54 t 69 Co se t f am (h f m [illegible] l 1⁄6 se d) f m phase t t m t p t re sequ a ph l h w g t t d g 1 dropl t t th pl m Th d pl t d sappe mm d t ly po t g th pl m E tru beg t bo t GU F 59 d mpl t d rta ly by FIGURE 65 Th d pl t trud d d t fi d by w U 54









P re 8

F r 70 t 85 C secut f am (h f am q l l se d) from ph se t t m t p t re seq ce m ocyt h w t t d g l d pl t to th plasm Th d pl t trud d f m the und th f f h ll It lose t id t ty mm d tely po t t th pla m Th dr pl t to be trud d d t h d by w 0 E tru io beg t bo t GU 7 d mpl ted f tain by F 85

DEPRESSIVE INFLUENCES ON LEUKOCYTIC NUMBERS

By Frank J Heck

Section of Medicine Mayo Clinic and Mayo Foundation Rochester Minn *

A decrease in the total number of leukocytes may be brought about by a wide variety of conditions My concern is primarily with the drugs which have been described as causing a reduction in the total leukocyte count to less than normal levels and with this decrease commonly a reduction in neutrophils I shall refer only briefly to other causes of leukopenia giving examples of these causes but without making any attempt to include all of them

There are many causes of leukopenia If one attempts to list the instances of leukopenia on the basis of their frequency then (1) the infectious causes would certainly head the list These causes could be subdivided into the more specific types of infections, such as (a) bacterial of which typhoid fever would be a good example (b) viral infections such as measles influenza or infectious hepatitis (c) protozoal infections such as malaria and (d) overwhelming infections such as septicemia or miliary tuberculosis Other causes of leukopenia are as follows (2) hematopoietic disorders or diseases involving particularly the liver and spleen (pernicious anemia in relapse leukopenic leukemias aplastic anemia portal hypertension cirrhosis of the liver splenic neutropenia), (3) a group of diseases of unknown cause (disseminated lupus erythematosus Felty s syndrome) (4) cachectic states and inanition (anaphylactoid shock) and (5) secondary effects of chemicals and physical agents In the cases listed in groups 1 to 4 the leukopenia is secondary to the disease whereas in group 5 which now includes a large number of chemicals we are dealing with a man made condition which in many instances if not promptly recognized may lead to the death of the patient This group of cases is therefore of particular importance from the clinical standpoint

Leukopenic Drugs

Under the name of agranulocytic angina Schultz [1] in 1922 reported a group of cases which were characterized by acute onset chills fever and ulceration of mucous membrane which were associated with an extreme leukopenia and neutropenia commonly terminating fatally Rare cases had been reported previously particularly one case by Brown [2] After 1922 there was a rapid succession of reports in both Germany and America describing cases of this type

These groups of cases were in contrast to the cases of benzol poisoning described earlier by Selling [3] in which the clinical course was much more chronic the leukopenia and granulocytopenia were accompanied by anemia and thrombocytopenia and at necropsy a hypoplastic marrow was found Selling followed this observation by experimentally producing leukopenia in rabbits by injection of benzene Similarly it has been noted that arsenic in both inorganic and organic form might give rise to a leukopenia

* The Mayo F[illegible] part of the Graduate School of the University of Minnesota Rochester Minn

As the result of clinical and pathologic observations made by Kracke in collaboration with Roberts[4] and after a very thorough survey of all the case reports available Kracke became impressed with the idea that the cases of acute granulocytopenia such as were described by Schultz[1] and Friedemann[5] were secondary to the ingestion of coal tar derivatives

In 1932 Kracke[6] published the results of work on the experimental production of agranulocytosis with benzene as well as with a number of chemicals said to be the oxidation products of benzene in the body (phenol paracresol orthocresol pyrocatechin hydroquinone ortho-oxybenzoic acid and paraoxybenzoic acid resorcinol) and several others With benzene he was able to reproduce in rabbits the clinical picture of agranulocytosis A marked leukopenia was produced by the subcutaneous injection of ortho-oxybenzoic acid (salicylic acid) and the intravenous injection of hydroquinone In a study of nine patients having agranulocytosis seen by him eight had been taking coal tar derivatives prior to the clinical onset However by oral administration of phenacetine aminopyrine peralga and dial to rabbits over a 45-day period no reduction in leukocyte count was obtained

Kracke did not carry out the crucial experiment of administering these drugs to his patients with agranulocytosis It remained for Madison and Squier[7] to show a specific relationship between agranulocytosis and the administration of aminopyrine All six of their patients in whom aminopyrine was used therapeutically after the onset of the disease died Of eight others in whom the drug was discontinued only two died Madison and Squier were able to induce typical attacks of agranulocytosis by the administration of aminopyrine Similar findings were reported simultaneously by other observers (Videbech[8] and Holten and his associates[9]) following which the relationship of the drug to the acute form of granulopenia became much better recognized and it was appreciated that many other drugs were also involved as etiologic agents

In 1934 Kracke and Parker[10] reported further on their experimental work and also gave data from the United States Bureau of Vital Statistics showing that in 1931 1932 and 1933 a total of 1 314 deaths was reported under the heading of agranulocytosis and agranulocytic angina

In the intervening years more than 50 different drugs (TABLE 1) have come to be recognized which may produce the syndrome variously described as agranulocytic angina agranulocytosis acute granulocytopenia or malignant neutropenia In addition to the foregoing however there are drugs which produce leukopenia and neutropenia of a much more chronic form and without specifically related clinical symptoms

As one reviews the literature it becomes apparent that these drugs can be classified to a certain extent in definite groups which may be quite closely related chemically While Kracke s original concept that benzene oxidation products were responsible for the disease has not been upheld yet the majority of drugs producing the disease are benzene ring or commonly some other type of ring compounds Although from the pharmacologic standpoint there is no specific relationship between structure and toxicity it is recognized that the introduction of certain chemical groups may have a modifying effect sufficient to make a compound much less toxic An example of this effect would be ac

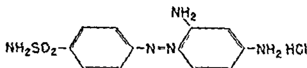
TABLE 1
DRUGS PRODUCING LEUKOPENIA

Sulfonamides Prontosil Neoprontosil Sulfanilamide Sulfapyridine Sulfathiazole Sulfaguanidine Sulfadiazine Sulfamerazine Succinyl ulfathiazole Gantrisin	*Organic arsenicals* Acetarsone Arsphenamine Mapharsen Neoarsphenamine Sulfarsphenamine
Drugs used in blood dyscrasias Aminopterin A methopterin 6 Mercaptopurine Nitrogen mustards Phenylhydrazine Triethylene melamine Urethan	*Miscellaneous* Pronestyl Tibione Atabrine Diparcol DDT Presidon
Benzene and simple derivatives Benzene Aniline Dinitrophenol	*Analgesic drugs* Antipyrine Aminopyrine Phenylbutazone Phenacetin
Antibiotics Chloramphenicol Penicillin Streptomycin	*Inorganic radicals* Arsenic Bismuth Gold Iron (IV) Mercury
Antihistamines Antergan Pyribenzamine Diatrin	*Antithyroid drugs* Thiourea Thiouracil Propylthiouracil Methylthiouracil Tapazole
Quinoline ring compounds Quinine Cincophen Vioform	*Anticonvulsant drugs* Dilantin Mesantoin Tridione Phenurone

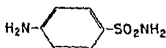
etanilid in which the substitution of an acetyl group for one of the hydrogens of the amino group in aniline markedly reduces the toxicity of the preparation Likewise it has been shown that an ethyl group substituted in place of a methyl group provides a preparation of lessened toxicity

While the toxicity of benzene and its simple derivatives phenol and aniline is well recognized there are so far as I am able to discover no authenticated reports of granulocytopenia or bone marrow damage resulting from such simple derivatives as salicylic acid or acetylsalicylic acid

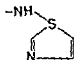
In addition to organic compounds there is also evidence of metallic elements having an adverse effect on leukocytes and the bone marrow The number of these elements is rather small and the compounds may be either inorganic or organic Up to the present time arsenic bismuth gold and mercury have been incriminated excluding those elements which depend for their leukocyte depressing effect on radiation of some type



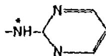
Prontosil



Sulfanilamide



Sulfathiazole



Sulfadiazine

FIGURE 1. Some of the members of the sulfonamide group.

In sheer numbers alone the various sulfonamide drugs (FIGURE 1) form the largest group of compounds known to produce agranulocytosis. Historically it is interesting to review the literature of the various compounds as they appeared and to note the statements made regarding hematopoietic effect. A group of 100 patients treated with a given drug would be reported and the statement would be made that there was no adverse effect on the blood only to be followed shortly by reports of agranulocytosis from the same drug. This phenomenon points up the necessity for recognizing that, for the most part, the occurrence of granulocytopenia or, for that matter, any other blood change secondary to drug ingestion is usually a rarity and that experience must be obtained not from merely 100 cases but rather from thousands before any broad statements regarding toxicity are made.

The various analgesics, aminopyrine, acetophenetidin, and antipyrine, form another large group to which there is occasional sensitivity. As noted previously, it was Kracke's observation that benzene ring compounds should be involved which led to a solution of this problem. As a part of his facts Kracke noted the increased frequency of occurrence of agranulocytosis in Germany and in the United States and its rarity in England, where this type of drug was used to a lesser extent. In the recent renewal of interest in drugs such as irgapyrin, butapyrin, and finally butazolidin, there was some tendency in England to discount the side effects of aminopyrine, and only after the report of unfavorable experiences was more adequate recognition given the possible dangers that this group of drugs might cause. In irgapyrin and butapyrin, which are mixtures containing aminopyrine, agranulocytosis was to be expected. Clinical observation showed that the closely related butazolidin also produced agranulocytosis. Another member of this group, 4-aminoantipyrine, has also been reported as producing agranulocytosis. FIGURE 2 shows the structural formulas of several of these drugs and their close chemical relationship.

The following cases demonstrate vividly the occasional leukopenic effect of

TABLE 1
DRUGS PRODUCING LEUKOPENIA

Sulfonamides	*Organic arsenicals*
Prontosil	Acetarsone
Neoprontosil	Arsphenamine
Sulfanilamide	Mapharsen
Sulfapyridine	Neoarsphenamine
Sulfathiazole	Sulfarsphenamine
Sulfaguanidine	
Sulfadiazine	*Miscellaneous*
Sulfamerazine	Pronestyl
Succinylsulfathiazole	Tibione
Gantrisin	Atabrine
	Diparcol
Drugs used in blood dyscrasias	DDT
Aminopterin	Presidon
A methopterin	
6 Mercaptopurine	*Analgesic drugs*
Nitrogen mustards	Antipyrine
Phenylhydrazine	Aminopyrine
Triethylene melamine	Phenylbutazone
Urethan	Phenacetin
Benzene and simple derivatives	*Inorganic radicals*
Benzene	Arsenic
Aniline	Bismuth
Dinitrophenol	Gold
	Iron (IV)
Antibiotics	Mercury
Chloramphenicol	
Penicillin	*Antithyroid drugs*
Streptomycin	Thiourea
	Thiouracil
Antihistamines	Propylthiouracil
Antergan	Methylthiouracil
Pyribenzamine	Tapazole
Diatrin	
	Anticonvulsant drugs
Quinoline ring compounds	Dilantin
Quinine	Mesantoin
Cincophen	Tridione
Vioform	Phenurone

etanilid in which the substitution of an acetyl group for one of the hydrogens of the amino group in aniline markedly reduces the toxicity of the preparation. Likewise it has been shown that an ethyl group substituted in place of a methyl group provides a preparation of lessened toxicity.

While the toxicity of benzene and its simple derivatives, phenol and aniline, is well recognized, there are, so far as I am able to discover, no authenticated reports of granulocytopenia or bone marrow damage resulting from such simple derivatives as salicylic acid or acetylsalicylic acid.

In addition to organic compounds, there is also evidence of metallic elements having an adverse effect on leukocytes and the bone marrow. The number of these elements is rather small, and the compounds may be either inorganic or organic. Up to the present time arsenic, bismuth, gold, and mercury have been incriminated, excluding those elements which depend for their leukocyte depressing effect on radiation of some type.

TABLE 2
BLOOD COUNTS IN CASE 1

Date	Leukocytes		
	Total per cu mm	Neutrophils, per cent	Eosinophils, per cent
September 29	1 400		
September 30	600	0	30
October 1	1 400	0	8
October 2	900	0	4
October 3	1 800	0	19
October 4	1 050	0	14
October 5	1 400	4	2
October 6	1 600	2	
October 7	1 100	14	
October 8			
October 9	1 300	14	
October 10	1 600	19	
October 14	2 600	21	
October 15	2 400	20	

Bone marrow aspiration disclosed on October [illegible] only [illegible] progranulocytes and myeloblasts [illegible] October 8 9 per cent my[illegible] and 2 per cent blasts October 15 marked increase in myeloid elements [illegible] October 8

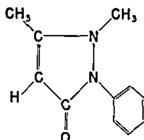
TABLE 3
BLOOD COUNTS IN CASE 2

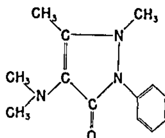
Date	Leukocytes		
	Total per cu mm	Polymorphonuclear cells, per cent	Immature myeloid cells, per cent
July 5	600	0	
July 6	800	0	
July 7	1 000	0	
July 8	300	0	
July 9	1 300	2	
July 10	2 200	18	10 5
July 11	2 900	40	14
July 12	4 100	17	28
July 14	9 400	58	1

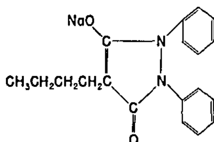
Bone marrow [illegible]

Members of two other groups of ring compounds that produce agranulocytosis are the antithyroid drugs and the anticonvulsant drugs The antithyroid drugs are derivatives of thiourea while the anticonvulsant drugs contain hydantoin or oxazolidine rings These drugs are shown in FIGURES 3 and 4

The last large group of drugs closely related chemically that produce agranulocytosis are the organic arsenicals shown in FIGURE 5 At the time when use of these preparations was at its peak pure agranulocytosis due to them was relatively rare and it was much commoner to see more severe hematologic disturbances such as aplastic anemia and thrombocytopenic purpura In the case of sulfarsphenamine the incidence of hematologic complications was so high that its use was abandoned in favor of the other safer arsenicals At pres-







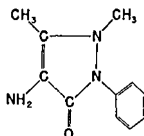


FIG. 2. Pyrazolone compounds.

these drugs.

Case 1. A male executive, aged 56 years, was seen Sept. 29, 1952, because of rheumatoid arthritis of 20 years' duration. Since August 18, when the administration of cortisone had been discontinued, he had been unusually fatigued and had been taking three tablets of phenylbutazone (butazolidin) daily. He had continued taking the latter drug through September 29, omitting it only on September 26. On September 30, the patient said he felt as though he had "flu." More fatigue and aching had started on the afternoon of September 29. The temperature was 101.2 F; there was no sore throat; the leukocytes numbered 600 per cubic millimeter of blood on September 30, and there were no polymorphonuclear leukocytes present. The blood counts for succeeding days are given in TABLE 2.

Case 2. A housewife, aged 28 years, was given four capsules of 4-aminoantipyrine daily for two months in the hope that it would relieve her symptoms of rheumatoid arthritis. On July 1, 1952, sore throat, sore gums, and blisters on the tongue developed. On July 2, the cervical lymph nodes were enlarged and the temperature rose to 102.6 F. On July 3, the leukocyte count was 2,100 per cu. mm. of blood with practically no polymorphonuclear leukocytes. Physical examination disclosed marked gingival hypertrophy and redness and moderate bilateral cervical adenopathy. Only protective treatment was given, consisting of 600,000 units of penicillin a day. The blood findings for succeeding days are given in TABLE 3.

TABLE 2
BLOOD COUNTS IN CASE 1

D t	Le kocyt		
	T t l pe mm	N t ophils, pe t	E ph l pe t
September 29	1 400		
September 30	600	0	30
October 1	1 400	0	8
October 2	900	0	4
October 3	1 800	0	19
October 4	1 050	0	14
October 5	1 400	4	2
October 6	1 600	2	
October 7	1 100	14	
October 8			
October 9	1 300	14	
October 10	1 600	1)	
October 14	2 600	21	
October 15	2 400	20	

B m rr w p rat d losed Oct be 1 ly tt ed p ogra locyt 1 my l bla t Oct be 8 9 p c t my l f ll d 2 pe c t bl t O t be 15 ma ked se in my l id l m t Oct be 8

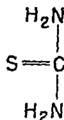
TABLE 3
BLOOD COUNTS IN CASE 2

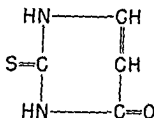
D t	Le kocyt		
	T t l pe mm	P lym rph ll pe t	Imm t my l d ll pe t
July 5	600	0	
July 6	850	0	
July 7	1 000	0	
July 8	900	0	
July 9	1 300	2	
July 10	2 200	18	10 5
July 11	2 900	40	14
July 12	4 100	17	28
July 14	9 400	58	17

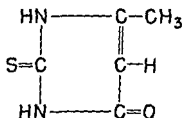
B marrow p t

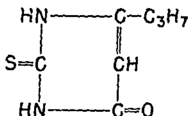
Members of two other groups of ring compounds that produce agranulocytosis are the antithyroid drugs and the anticonvulsant drugs The antithyroid drugs are derivatives of thiourea while the anticonvulsant drugs contain hydantoin or oxazolidine rings These drugs are shown in FIGURES 3 and 4

The last large group of drugs closely related chemically that produce agranulocytosis are the organic arsenicals shown in FIGURE 5 At the time when use of these preparations was at its peak pure agranulocytosis due to them was relatively rare and it was much commoner to see more severe hematologic disturbances such as aplastic anemia and thrombocytopenic purpura In the case of sulfarsphenamine the incidence of hematologic complications was so high that its use was abandoned in favor of the other safer arsenicals At pres-









F URE 3 A t thyr d d ugs (th ou and de at e)

ent with the marked reduction in use of all arsenicals reactions of this kind are extremely rare

Certain quinoline compounds and a host of other not necessarily related benzene ring compounds and other organic compounds comprise the greater percentage of drugs not grouped above These drugs include such diversified preparations as some of the antihistamine preparations Aminopterin nitrogen mustard procaine amide hydrochloride (pronestyl hydrochloride) and others

In the metallic element group my colleagues and I have recently experienced a case which while unproved as to the exact compound involved was almost certainly secondary to the ingestion of inorganic arsenic In contrast to the acute agranulocytosis produced by most of the members of this group this case was the type of chronic leukopenia which is seen following administration of nitrogen mustard gold or radiation therapy and other agents In most of the more chronic leukopenias there are seldom any clinical symptoms present which would direct attention to a consideration of the leukocyte count and recognition comes about only as the result of routine blood studies Lawson Jackson and Cattanach [11] in 1925 reported a series of cases of arsenic poisoning in which there were leukopenia and eosinophilia with rapid return over a period of several weeks to a normal total and differential count

Case 3 The patient a white man aged 41 years registered April 7 1954 complaining of paresthesias in arms and legs and inability to walk About Feb

Dilantin

Mesantoin

Furadantin
N-(5-nitro-2 furfurylidene)-
1-aminohydantoin

Tridione
3 5 5 trimethyl-
oxazolidine 2 4 dione

F g 4 Se ral a t lsa t drugs F d t hown abo ls n t a ticon ulsa t drug but s l d d bec use t hyda t d t It h t been report d s prod cing granulocytopen a.

Acetarsone

Neoarsphenamine

Maphars n

Sulpharsphenamine

F g 5 Som m mbe f th g c rse cal g up

1 1954 he had noted constant dull left frontal headache, starting at noon and lasting the rest of the day The headache would be gone the next morning On March 6 following ingestion of braunschweiger sausage, he felt nauseated On March 7, he vomited before breakfast He went to work but became progressively more fatigued during the day and was unable to work On March 9 his eyes were sore and red and periorbital puffiness developed Exhaustion and weakness continued through this period On March 29 the leukocyte count was found to be 450 per cu mm of blood and on March 30 550 per cu mm At this time numbness and tingling in tips of fingers and toes developed The patient could not walk without help because his knees and ankles gave way Paresthesias rapidly extended to above the knee and to the wrist Between March 31 and April 5 the leukocyte count varied between 1 150 and 3 250 per cu mm of blood with the eosinophil percentage 21

On physical examination the outstanding finding was weakness of muscles of upper and lower extremities with complete loss of joint sense in feet, ankles and knees and moderate reduction of joint sense in fingers There was marked reduction of vibratory sensation over ankles and hands Lumbar puncture did not show a diagnostic change Because of the picture of a motor and sensory neuritis the urine was examined for arsenic and showed a concentration of 1 65 mgm per liter Subsequently fingernails and hair were also checked for arsenic with values reported of 2 58 mgm per 100 gm and 1 76 mgm per 100 gm respectively Initial leukocyte count was 2 600 per cu mm of blood with 19 per cent eosinophils A diagnosis of arsenical neuritis was made and treatment with BAL was instituted on April 12 By April 23 the leukocyte count was 5 400 per cu mm of blood and the differential count showed lymphocytes 27 per cent neutrophils 70 per cent and eosinophils 3 per cent Bone marrow aspiration on April 13 showed a somewhat hypocellular marrow but with all marrow elements present in about normal distribution

Mode of Action

Madison and Squier at the time of their original article in 1934 felt that in view of the widespread use of such drugs as aminopyrine and the very rare occurrence of cases of agranulocytosis an allergic or hypersensitivity reaction would offer the best explanation for the development of the illness Dameshek and Colmes[12] were unable to reproduce the symptomatology in patients recovered from the disease by the intradermal injection of a plain solution of aminopyrine but showed that a blood serum aminopyrine mixture which had been aged in the icebox several days and then injected intradermally produced the syndrome They accordingly postulated a drug protein linkage as the basis of an allergic or hypersensitive reaction

In 1952 Moeschlin and Wagner[13] reported their study in which 300 cc of blood was taken from a patient with aminopyrine sensitivity after 0 3 gm of aminopyrine had been given and this blood was transfused into a normal person A marked drop occurred in the total leukocyte count of the recipient the count decreasing from 5 000 to 800 per cubic millimeter of blood after 40 minutes The recipient experienced subjective discomfort and became pale though no fever or shaking chill appeared The decrease in leukocytes occurred

mainly in the granulocytes and there was a return to a normal count within four hours. Simple administration of aminopyrine to the recipient did not produce any change in the number of leukocytes The study was repeated in another recipient with the same result In studies *in vitro* Moeschlin and Wagner showed agglutination of normal leukocytes as well as of the leukocytes of an aminopyrine-sensitive patient when these were mixed with serum or plasma of an aminopyrine-sensitive person three hours after administration of 0.3 gm of aminopyrine They therefore postulated the production in sensitive patients after administration of aminopyrine of a factor that causes agglutination of granulocytes They theorized on the basis of the work of Weisberger Heinle and Hannah [14] that the agglutinated leukocytes are removed mainly by the lung

It has been established by Rohr[15] that in the bone marrow there is to a large extent a loss of cells from mature to less mature forms and that the degree of loss is related to the amount of drug administered In other words the metamyelocytes tend to disappear first and later the myelocytes and finally in severe cases only promyelocytes or even reticulum cells remain These effects correspond to what is observed generally in the disease in that recovery is much less likely to occur if administration of the offending drug is continued The peripheral destruction and loss of leukocytes lead to increased demand on the marrow with resulting exhaustion of the marrow Whether this interpretation is entirely correct or not the facts as seen clinically fit in very well with such a concept

It therefore seems likely that almost all cases of acute primary granulocytopenia (that is where there is no other disease present causing a drop in leukocytes) should be explicable on such a basis One other bit of evidence might be brought up in support of the allergic or sensitivity theory Christensen[16, 17] has shown that there is a high percentage of protein binding for a number of the thiouracils although in the most active compounds the degree of protein binding was the least so that in the case of the antithyroid drugs one might expect a higher proportion of patients treated with these drugs to develop agranulocytosis than of those treated by other methods

A second method by which cells and bone marrow could be affected would be by a toxic change the method postulated for gold benzol urethane and so forth where although acute reactions occur a more chronic reduction of the leukocytes is more often seen Bastrup-Madsen[18] classified cytotoxic compounds into three main groups (1) specific mitotic compounds (spindle poisons) of which colchicine is typical (2) agents with an unspecified cytotoxic effect to which group arsenical and urethane belong and (3) agents with a radiomimetic effect Nitrogen mustard belongs to this group and affects cells in the resting phase This effect cannot be noted until the cells divide after having been exposed to the injurious agent

Summary

(1) Two types of reactions are seen secondary to administration of drugs which produce a leukopenia and loss of neutrophils and at times of lymphocytes (a) agranulocytosis in which there is acute reaction with chills fever

1, 1954 he had noted constant dull left frontal headache, starting at noon and lasting the rest of the day The headache would be gone the next morning On March 6 following ingestion of braunschweiger sausage, he felt nauseated On March 7, he vomited before breakfast He went to work but became progressively more fatigued during the day and was unable to work On March 9, his eyes were sore and red and periorbital puffiness developed Exhaustion and weakness continued through this period On March 29, the leukocyte count was found to be 450 per cu mm of blood and on March 30 550 per cu mm At this time numbness and tingling in tips of fingers and toes developed The patient could not walk without help because his knees and ankles gave way Paresthesias rapidly extended to above the knee and to the wrist Between March 31 and April 5 the leukocyte count varied between 1,150 and 3 250 per cu mm of blood with the eosinophil percentage 21

On physical examination the outstanding finding was weakness of muscles of upper and lower extremities with complete loss of joint sense in feet ankles and knees and moderate reduction of joint sense in fingers There was marked reduction of vibratory sensation over ankles and hands Lumbar puncture did not show a diagnostic change Because of the picture of a motor and sensory neuritis the urine was examined for arsenic and showed a concentration of 1 65 mgm per liter Subsequently fingernails and hair were also checked for arsenic with values reported of 2 58 mgm per 100 gm and 1 76 mgm per 100 gm respectively Initial leukocyte count was 2 600 per cu mm of blood with 19 per cent eosinophils A diagnosis of arsenical neuritis was made, and treatment with BAL was instituted on April 12 By April 23 the leukocyte count was 5 400 per cu mm of blood and the differential count showed lymphocytes 27 per cent neutrophils 70 per cent and eosinophils 3 per cent Bone marrow aspiration on April 13 showed a somewhat hypocellular marrow but with all marrow elements present in about normal distribution

Mode of Action

Madison and Squier at the time of their original article in 1934 felt that in view of the widespread use of such drugs as aminopyrine and the very rare occurrence of cases of agranulocytosis an allergic or hypersensitivity reaction would offer the best explanation for the development of the illness Dameshek and Colmes[12] were unable to reproduce the symptomatology in patients recovered from the disease by the intradermal injection of a plain solution of aminopyrine but showed that a blood serum aminopyrine mixture which had been aged in the icebox several days and then injected intradermally produced the syndrome They accordingly postulated a drug protein linkage as the basis of an allergic or hypersensitive reaction

In 1952 Moeschlin and Wagner[13] reported their study in which 300 cc of blood was taken from a patient with aminopyrine sensitivity after 0 3 gm of aminopyrine had been given and this blood was transfused into a normal person A marked drop occurred in the total leukocyte count of the recipient the count decreasing from 5 000 to 800 per cubic millimeter of blood after 40 minutes The recipient experienced subjective discomfort and became pale though no fever or shaking chill appeared The decrease in leukocytes occurred

mainly in the granulocytes and there was a return to a normal count within four hours Simple administration of aminopyrine to the recipient did not produce any change in the number of leukocytes The study was repeated in another recipient, with the same result In studies *in vitro* Moeschlin and Wagner showed agglutination of normal leukocytes, as well as of the leukocytes of an aminopyrine sensitive patient when these were mixed with serum or plasma of an aminopyrine sensitive person three hours after administration of 0 3 gm of aminopyrine They therefore postulated the production in sensitive patients after administration of aminopyrine, of a factor that causes agglutination of granulocytes They theorized on the basis of the work of Weisberger Heinle and Hannah,[14] that the agglutinated leukocytes are removed mainly by the lung

It has been established by Rohr[15] that in the bone marrow there is to a large extent a loss of cells from mature to less mature forms and that the degree of loss is related to the amount of drug administered In other words the metamyelocytes tend to disappear first and later the myelocytes and finally in severe cases only promyelocytes or even reticulum cells remain These effects correspond to what is observed generally in the disease in that recovery is much less likely to occur if administration of the offending drug is continued The peripheral destruction and loss of leukocytes lead to increased demand on the marrow, with resulting exhaustion of the marrow Whether this interpretation is entirely correct or not the facts as seen clinically fit in very well with such a concept

It therefore seems likely that almost all cases of acute primary granulocytopenia (that is where there is no other disease present causing a drop in leukocytes) should be explicable on such a basis One other bit of evidence might be brought up in support of the allergic or sensitivity theory Christensen[16, 17] has shown that there is a high percentage of protein binding for a number of the thiouracils although in the most active compounds the degree of protein binding was the least so that in the case of the antithyroid drugs one might expect a higher proportion of patients treated with these drugs to develop agranulocytosis than of those treated by other methods

A second method by which cells and bone marrow could be affected would be by a toxic change the method postulated for gold benzol urethane, and so forth where although acute reactions occur a more chronic reduction of the leukocytes is more often seen Bastrup-Madsen[18] classified cytotoxic compounds into three main groups (1) specific mitotic compounds (spindle poisons) of which colchicine is typical (2) agents with an unspecified cytotoxic effect to which group arsenicals and urethane belong and (3) agents with a radiomimetic effect Nitrogen mustard belongs to this group and affects cells in the resting phase This effect cannot be noted until the cells divide after having been exposed to the injurious agent

Summary

(1) Two types of reactions are seen secondary to administration of drugs which produce a leukopenia and loss of neutrophils and at times of lymphocytes (a) agranulocytosis in which there is acute reaction with chills fever

mucosal ulceration and, commonly, bone pain, (b) a more chronic type of leukopenia with neutropenia, which, in the main, is not productive of the clinical syndrome described under (a)

(2) *More than 50 drugs are now known to produce acute reactions with* agranulocytosis These drugs may fall into several groups the members of which are closely related chemically, such as the sulfonamides, certain analgesic drugs, organic arsenicals, antithyroid, and anticonvulsant groups Many of the others show some chemical relationship Not all these drugs contain the benzene ring but *almost all of them are ring compounds of one type or another*

(3) The acute reactions are best explained on an allergic or sensitivity basis The evidence for this viewpoint is presented

(4) The drugs producing the more chronic type of reactions commonly do so as the result of a toxic effect on the marrow cells

References

1 SCHULTZ W 1922 Ueber eigenartige Halserkrankungen Deut med Wochschr 48 1495–1496
2 BROWN P K 1902 A fatal case of acute primary infectious pharyngitis with extreme leukopenia Am Med 3 649–651
3 SELLING L 1916 Benzol as a leucotoxin Studies on the degeneration and regeneration of the blood and haematopoietic organs Johns Hopkins Hosp Rept 17 83–142
4 ROBERTS S R & R R KRACKE 1931 Agranulocytosis Its classification cases and comments illustrating the granulopenic trend from 8 000 blood counts in the South Ann Internal Med 5 40–51
5 FRIEDEMANN U 1923 Über Angina agranulocytotica Med Klin 19 1357–1359
6 KRACKE R R 1932 The experimental production of agranulocytosis Am J Clin Path 2 11–30
7 MADISON F W & T L SQUIER 1934 The etiology of primary granulocytopenia (Agranulocytic angina) J Am Med Assoc 102 755–759
8 VIDEBECH H 1933 Tilfaede af agranulocytose med exanthem Hospitalstidende 76 535–539
9 HOLTEN C H E NIELSEN & K TRANSBØL 1934 Five nosocomial cases of agranulocytosis in patients treated with amidopyrine Contribution to knowledge of etiology of agranulocytosis (Preliminary rept) Ugeskrift laeger 96 155–158
10 KRACKE R R & F P PARKER 1934 The etiology of granulopenia (Agranulocytosis) With particular reference to drugs containing the benzene ring Am J Clin Path 4 453–469
11 LAWSON G B W P JACKSON & G S CATTANACH 1925 Arsenic poisoning Report of twenty eight cases J Am Med Assoc 85 24–26
12 DAMESHEK W & A COLMES 1936 The effects of drugs in the production of agranulocytosis with particular reference to amidopyrine hypersensitivity J Clin Invest 15 85–97
13 MOESCHLIN S & K WAGNER 1952 Agranulocytosis due to the occurrence of leukocyte agglutinins (Pyramidon and cold agglutinins) Acta haemat 8 29–41
14 WEISBERGER A S R W HEINLE & R HANNAH 1949 Transfusion of leukocytes and products of disintegrated leukocytes Proc Exptl Biol Med 70 749–753
15 ROHR K 1936 Blut und Knochenmarksmorphologie der Agranulocytosen (Ergebnisse fortlaufender Sternalmarkuntersuchungen) Folia haemat 55 305–367
16 CHRISTENSEN H N 1945 Ultrafiltrability of thiouracil in human serum determination of thiouracil J Biol Chem 160 425–433
17 CHRISTENSEN H N 1946 Analytical determination and some properties of several thyroid inhibiting compounds and of substances related to them J Biol Chem 162 27–35
18 BASTRUP-MADSEN P 1951 Action of some antineoplastic chemotherapeutical agents on cell division Acta Radiol 36 452–460

SOME ASPECTS OF HORMONAL INFLUENCES UPON THE LEUKOCYTES*

By Albert S Gordon

Department of Biology Graduate School of Arts and Science New York University New York N Y

The physiologist engaged in hematologic research constantly seeks to uncover new approaches toward the solution of one of his most elusive problems the functions of the blood leukocytes It has been thought that this question might be treated through a consideration of the mechanisms by which environ mental stimuli influence the production distribution and ultimate fate of the leukocytes It was felt that a collation of the essential facts gathered from such a discussion might stimulate new interest reveal clues and provide spring boards for the launching of more vigorous attacks on problems linked directly to leukocytic physiology

Evidence has been steadily accumulating in recent years to show that the endocrine factors must be added to the list of potent stimuli that exert an in fluence upon the white blood elements It will be the purpose of this report to review briefly some of the more pertinent evidence favoring an endocrine relation to the leukocytes Emphasis will be placed upon those phases dealing with the pituitary and adrenal glands which have been the subject of recent intensive attack

A relation between the ductless glands and the blood leukocytes had been in timated in many of the early clinical reports Illustrative of this are the lym phocytosis characteristic of the Addisonian[1] the eosinophilia of Simmonds disease[2] and the lymphocytosis and granulocytopenia forming a part of Kocher s[3] blood picture in thyrotoxicosis Apart from a few experimental reports[4-7] describing lymphocytosis and neutropenia as characteristic of hypoadrenalism *generalized interest in this field lay relatively dormant until the* appearance of evidence demonstrating the unequivocal participation of the pituitary adrenal axis in the regulation of the blood leukocytes Selye s[8] basic description of lymphoid tissue involution as an accompaniment to the alarm reaction in stress set the stage for further and more detailed studies of endocrine influences upon the lymphocytes The experiments of Dalton and Selye[9] revealed lym phopenia eosinopenia and neutrophilia as a triad of peripheral blood effects in animals stressed by formaldehyde injections or by severe exercise Dough erty and White[10] demonstrated that administration of a single injection of ACTH into mice rats and rabbits induced, within a few hours absolute lym phopenia and neutrophilia The lymphopenic response to ACTH was abol ished by adrenal removal but not by hypophysectomy On the other hand adrenal cortical extracts and 11 oxysteroids were able to produce a depression of lymphocytes not only in intact animals but in those deprived of their ad renals as well The studies of Hills Forsham and Finch[11] served to delineate

S m f th pe m tal w k d sc bed th po t w d t d th tho labo t ry d n g th f g t d f m th Am ca Ca S ty po mm dat f th Comm tt G wth f th N t l R se h C un l d b f d p d d d t t AF 18(600)366 w th th U ted State A F S hool f A iat M d R d lph F ld T xas

the influence of ACTH upon the formed elements of the peripheral blood of human subjects At four hours after the injection of 25 mgm of ACTH a mean decrease in lymphocytes of 39 per cent and a neutrophil rise of 104 per cent were reported Employing the chamber method for enumerating eosinophils a mean decrease of 73 per cent in these elements was detected in the same patients The absence of the lymphocyte and eosinophil response to ACTH in the Addisonian patient led to the inescapable conclusion that a functional adrenal cortex was necessary for the evocation of the reaction The clear cut difference noted in the eosinophil response to ACTH in the Addisonian as compared to the normal subject suggested the use of this test in the diagnosis of adrenocortical insufficiency[12] and as a bioassay for ACTH and the 11-oxysteroids[13, 14]

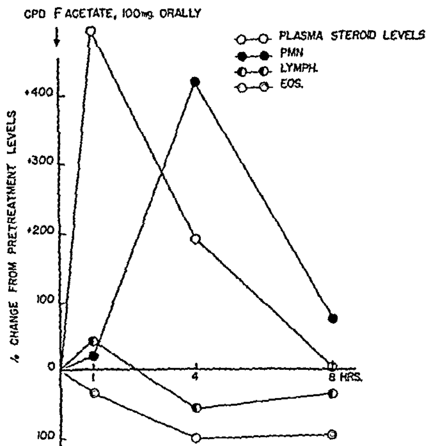
In order to visualize more clearly the influence of adrenal factors upon the leukocytes the data of Nelson *et al*[15] have been plotted on a single graph (FIGURE 1) Here are indicated the effects of a single 100 mgm dose of Compound F (17 hydroxycorticosterone acetate) administered orally to a human subject Within one hour there occurs a 35 per cent decrease in the numbers of circulating eosinophils accompanied by a slight rise in the numbers of lymphocytes and polymorphonuclear leukocytes At four hours eosinophils have disappeared from the peripheral blood a reaction associated with a significant decrease in the numbers of lymphocytes and a marked rise in the numbers of neutrophilic elements Slight waning of these effects is apparent by the eighth hour It will be observed that the blood levels of 17 hydroxycorticosteroids become maximal shortly before the peak deviations in the formed elements occur The total white cell count generally increases in animals that have a greater proportion of neutrophils to lymphocytes (*e g* in man) and decreases in forms such as the rabbit mouse and rat[10] in which the lymphocyte is the predominant circulating white blood cell

An attempt will be made now to analyze briefly the mechanisms underlying pituitary adrenal influences upon the peripheral white cells It should be apparent that the numbers of formed elements in the circulating body fluids are a reflection of the homeodynamic balance between those factors regulating the production and release of the cells from organs and tissue sites as opposed to those forces influencing their removal There is evidence available that many if not all of these phenomena are influenced by the endocrine system

I Lymphocytes

A *Is the Lymphopenia Induced by Adrenal Cortical Factors and Stress Due to Effects Exerted upon the Lymphatic Organs?*

Adrenalectomy has been reported uniformly to result in an increase in the absolute weight of the lymphoid tissues[16] Doubt however has been cast on the interpretation that this increase represents true hyperplasia because of evidence that the thymus/body weight ratios in adrenalectomized animals are no different from those noted in intact well fed animals of the same strain subjected to minimal stress[17] This observation is supported by the finding that no increase in the numbers of thymocytic mitoses occurs following adrenal



F[illegible] 1 Effect [illegible] [illegible]gle dose [illegible] hydrocortisone a[illegible]tate upon the plasma 17 hydroxy[illegible] [illegible]osteroid a[illegible]d leukocytic (polymorphon[illegible] [illegible]ne[illegible]trophils eos[illegible] [illegible] [illegible]d lymphocyt[illegible] [illegible] [illegible] [illegible] [illegible] subje[illegible]t (calculated on a pe[illegible]ent [illegible]h[illegible]nge ba[illegible] from the data of [illegible] [illegible])

ectomy in the rat[13] On the basis of the evidence one is inclined to agree with Selye[19] that adrenal removal serves merely to protect the lymphoid tissues against those influences that would produce involution

More impressive evidence for a pituitary adrenal action upon the lymphatic tissues is derived from studies dealing with the effects of various stresses and adrenal hormones It was Selye[19] who first crystallized the concept of lymphoid tissue involution during the alarm reaction phase of the general adaptation syndrome Selye demonstrated that a wide variety of alarming stimuli (epinephrine formaldehyde morphine atropine spinal shock surgical shock cold exercise) produce within a few hours marked disintegration of the lym phoid elements within the thymus Increased phagocytosis, edema of the

connective tissue stroma and hyperplasia of the reticulum are associated phenomena In the severe reaction only the stroma and modified reticulum remain and the thymus weight is greatly reduced The reaction of the lymph nodes and spleen is similar to but less intense than that displayed by the thymus Adrenalectomy was found to prevent the thymic involution that follows the application of noxious stimuli

Dougherty[16] has described succinctly the effects of ACTH and adrenal cortical factors upon the lymphatic organs Following a single injection of ACTH the earliest change in lymphatic tissue was a marked edema which occurred within one hour and persisted for six hours During the stage of edema the lymphocytes diminished in numbers and exhibited marked degenerative changes There was a cessation of mitosis of small and medium sized lymphocytes and a development of reaction centers in the lymph nodes Later (six to nine hours) numerous macrophages were observed which were filled with nuclear debris derived from disintegrating lymphocytes These latter cells showed no degenerative alterations with amounts of adrenocortical hormones which destroyed the smaller lymphocytes Histiocytes underwent various morphological alterations but showed no signs of degeneration The most marked degenerative effects were noted in the small and medium sized lymphocytes which displayed cytoplasmic budding pyknosis, and karyorrhexis

These descriptions emphasize a basic similarity in the pattern of response of the lymphatic organs to stress and to ACTH and therefore tend to implicate the adrenal cortex in the regulation of the numbers of circulating lymphocytes It should be emphasized that although the effects of stress appear to be mediated in large measure through the pituitary adrenal axis, there are examples of stressor agents which produce acute involution of the lymphoid tissues in the absence of the adrenal These agents include X radiation the nitrogen mustards and pyridoxine deficiency The data however are uniform in indicating that the effects of these agents in the intact animal must be attributed at least in part to involvement of endocrine phenomena

More details of the effects of adrenal steroids upon the lymphatic system have been provided by Santisteban and Dougherty [20] Kendall s Compound F (17 hydroxycorticosterone) was found to be the most potent in causing thymic involution in the adrenalectomized mouse with Compounds E (11 dehydro 17 hydroxycorticosterone) B (corticosterone) and A (11 dehydrocorticosterone) possessing a decreasing order of effectiveness Reichstein s Substances L [allopregnane 3(β) 17(α) diol 20-one] and S [4 pregnene 17(α) 21-diol-3 20 dione] were shown to have some thymolytic activity whereas DCA (11 desoxy corticosterone acetate) glucoside increased thymic weight Likewise, progesterone has been reported to increase thymic size and Substance L testosterone and estrogen to evoke hypertrophy of lymph nodes in the rat [21] It is of interest that adrenal cortical extracts more effective than single 11 oxysteroids in inducing weight gains in adrenalectomized animals are less effective in evoking thymic atrophy [16, 22] The possibility should be investigated that some of the steroids present in the whole extract may function in an antagonistic manner towards the lymphoid elements

With continuous administration of ACTH there results a marked suppres

sion of lymphocytic mitosis and therefore an impaired production of lymphocytes It is of interest that in such chronically treated rats lymphocytolysis is not seen as clearly as in the acutely treated animal[3] probably because fewer lymphocytes are available for destruction Emphasized in this report is the greater sensitivity to ACTH of the thymocytes over that of the lymphoid elements of the spleen and lymph nodes This greater sensitivity may be related to the greater rate of cellular proliferation in the thymic parenchyma[24]

It has been observed that the pattern of peripheral lymphocytic behavior evoked by stress is not the exact counterpart of that resulting from adrenal cortical treatment[-5] Following stress there occurs first a rise in the levels of circulating lymphocytes (Phase I) This rise is then followed by the characteristic lymphopenia (Phase II) after which there is a return to normal levels (Phase III) Epinephrine release is held to be the factor accountable for Phase I It is difficult to accept the hypothesis that this agent acts solely through stimulating the release of lymphocytes from the spleen since it can occur in the splenectomized animal

Not only is a full complement of lymphoid tissue involution in response to stressor agents dependent upon the persistence of some adrenal cortical function but an actual enhanced lymphopoiesis may occur in response to stress in the absence of the adrenal[26] Subjection of adrenalectomized mice to such stresses as starvation histamine epinephrine or anaphylactic shock results in a lymphocytosis of far greater magnitude than that seen in the unstressed adrenalectomized animal Splenectomy does not alter this response The cells contributing to this 'lymphocytotic response show an increased cytoplasmic nuclear ratio and a weakly basophilic cytoplasm These stress lymphocytes are said to resemble the cells seen in infectious mononucleosis (Type II lymphocytes of Downey)

B *Possible Direct Effects of Adrenal Factors upon the Lymphocyte*

Opinion is divided as to the *in vitro* effects of cortical steroids upon the lymphocytes Heilman[27] observed that the addition of cortisone to hanging drop cultures of lymph nodes led to inhibition of lymphocytic migration and to some augmentation in degeneration after 24 hours Similarly Holden Seegal and Ryby[28] noted an inhibitory action of cortisone acetate upon the migration of lymphocytes in spleen cultures On the other hand Baldridge *et al*[9] could find no evidence for any lymphocytolytic effect of cortisone acetate when added to hanging drop cultures of buffy coat of human blood Absence of direct lymphocytolytic action of aqueous adrenal extracts[30] and of Compound A (11 dehydrocorticosterone)[31] has also been reported On the other hand Schrek[32, 33] found shortened survival time (increased eosin staining) of lymphocytes incubated with small concentrations of Compounds F B or E Doses as small as 0 06 μg per ml were found to be effective Hechter and Johnson[34] and Feldman[3] observed that addition of cortical extracts increased the susceptibility of lymphocytes to lysis A number of 11 oxysteroids was also tested including Compounds E and B which were found incapable of accelerating lymphocytic destruction Feldman[36] has observed that incubation with an adrenal cortical extract (lipoadrenal cortex) results in a characteristic cyto-

plasmic bubbling in neutrophils lymphocytes, monocytes and eosinophils, a phenomenon preceding their disintegration Sex steroids were also found to exert a destructive influence upon the leukocytes which however, was not preceded by the cytoplasmic bubbling More recently, Frank and Dougherty[37] have reported a similar bubbling phenomenon in human lymphocytes in a buffy coat preparation suspended in plasma and exposed to hydrocortisone or cortisone Of considerable interest in this field are the recent experiments of Trowell[38] who studied the effects of adrenal steroids added directly to rat lymph nodes cultivated in a serum saline medium on wet cotton wool Cortisone exerted a definite destructive effect in quantities as small as 0 1 μg per ml The process apparently is a slow one, with the numbers of lymphocytes killed proportional to the log of time over the first 24 hours Desoxycorticosterone was slightly effective in larger concentrations whereas testosterone, estradiol and progesterone proved ineffective No specific cytologic alterations in the lymphocytes were apparent from which Trowell has concluded that the phenomenon of lymphocytic shedding and budding represents a manifestation of a generalized cell destruction process not peculiar to the lymphocyte Calculations indicate that the destructive action upon lymphocytes is observed with cortisone at a concentration lower than that seen with any other substance (*e g*, it is 1000 times more toxic than cyanide) It is of interest that the reticular cells and macrophages in the culture remained unaltered by the steroid addition

It should be clear although it is not always kept in mind that statements regarding a possible direct influence of hormonal agents are not warranted unless only the particular cell type in question is present in the experimental system This condition holds especially for tissue culture incubations not involving pure cellular types and even for buffy coat preparations in which more than one type is included It is conceivable that the effect of the hormone might be exerted directly upon another cellular line and that the secretory or breakdown products yielded by this element might then exert a secondary action upon the cell examined Experiments should be directed at determining the particular cell type that responds first either on a structural (histochemical) or metabolic basis, to the adrenocortical steroids for this finding might provide a clue as to the initial link in the chain of cellular effects induced by these factors Remaining to be determined in this connection, is the significance of the lymphopenic reaction preceding chronologically the eosinopenia following administration of pituitary adrenal agents[39]

In summary the evidence remains equivocal as to whether adrenal cortical factors exert a direct influence upon the lymphocytes in lymphatic organs or in the body fluids Further experiments are required to test the possibility that other tissues particularly reticuloendothelial in nature mediate the effects of the adrenal cortex and of stress in the destructive process

C *Possible Effects on Redistribution of Lymphocytes*

Indications can be found in the literature that endocrine factors influence the movement of lymphocytic elements from the circulating blood to organs where they may be temporarily sequestered Thus Yoffey[40] has deduced that the

lymphoid nodules found in bone marrow represent 'focal accumulations of hematogenous lymphocytes which have migrated through the walls of the capillaries Farr[41] employed lymphocytes treated with a diamino acridinium nontoxic vital stain which renders the cells fluorescent when stimulated with near ultraviolet light Following their intravenous injection such labeled lymphocytes were found within two hours in the bone marrow thymus mesenteric lymph node appendix Peyer s patches and the splenic white pulp According to Yoffey and his associates[42] the peripheral lymphopenia caused by a single injection of ACTH or cortical extract is accompanied by a significant increase in the numbers of lymphocytes within the bone marrow It was observed however that repeated administration of ACTH over a seven-day period resulted in no significant alteration in the numbers of marrow lymphocytes[43]

It is difficult to decide at present to what extent migration of the lymphocytes to tissue sites contributes to the acute peripheral lymphopenia induced by cortical factors or by stress Such a tendency might be obscured in some species by simultaneous release of lymphocytes from storage organs including the spleen as had been observed following administration of adrenal cortical extract[44, 45] Although the lung has also been implicated as a site of lymphocytic sequestration experiments by Weisberger and his associates[46] have shown that the rapid filtration of intravenously administered P_{32} labeled lymphocytes by the pulmonary system is not altered by ACTH or cortisone This finding however does not preclude the participation of other organs in the phenomenon

In summary it would seem that the lymphopenic state developing in the early stages of 11-oxysteroid or stress action derives to an extent from the destructive effects exerted directly or indirectly upon the small and medium sized lymphocytes within the lymphatic organs thereby resulting in a decreased delivery of these cells into the lymphatic[47, 49] and general circulation The extent of the contribution of this lymphocytolytic effect to the peripheral lymphopenia must await more definitive evidence regarding the longevity of the lymphocyte within the organism A possible operation of endocrine factors in eliciting the acute blood lymphopenia through altering the rate of migration of lymphocytes to a variety of body organs including the bone marrow liver kidney and lymphatic tissues must also be considered In this connection there is urgent need for the critical experiment that attempts to measure simultaneously the numbers of lymphocytes entering and leaving the suspected sequestering organs at various intervals of time following single and multiple injections of adrenal and other endocrine factors

When relatively large quantities of 11 oxysteroids or ACTH are given for a protracted period of time the predominant action would appear to consist in susceptible species of a reduction in the total lymphatic tissue mass both as a consequence of previously imposed destruction of lymphoid elements and through actual suppression of homoplastic and heteroplastic lymphocytopoiesis[20, 23]

With relatively small amounts of adrenal factors particularly adrenal cortical extracts[50] lymphatic escape and even hyperplasia may occur One wonders to what extent this effect may be the result of adaptation to the hormone or factor administered[51, 52] Since such steroids as desoxycorticos

plasmic bubbling in neutrophils lymphocytes, monocytes, and eosinophils a phenomenon preceding their disintegration Sex steroids were also found to exert a destructive influence upon the leukocytes which however was not preceded by the cytoplasmic bubbling More recently, Frank and Dougherty[37] have reported a similar bubbling phenomenon in human lymphocytes in a buffy coat preparation suspended in plasma and exposed to hydrocortisone or cortisone Of considerable interest in this field are the recent experiments of Trowell[38] who studied the effects of adrenal steroids added directly to rat lymph nodes cultivated in a serum saline medium on wet cotton wool Cortisone exerted a definite destructive effect in quantities as small as 0 1 μg per ml *The process, apparently, is a slow one with the numbers of lymphocytes killed* proportional to the log of time over the first 24 hours Desoxycorticosterone was slightly effective in larger concentrations whereas testosterone estradiol and progesterone proved ineffective No specific cytologic alterations in the lymphocytes were apparent from which Trowell has concluded that the phenomenon of lymphocytic shedding and budding represents a manifestation of a generalized cell destruction process not peculiar to the lymphocyte Calculations indicate that the destructive action upon lymphocytes is observed with cortisone at a concentration lower than that seen with any other substance (*e g* it is 1000 times more toxic than cyanide) It is of interest that the reticular cells and macrophages in the culture remained unaltered by the steroid addition

It should be clear although it is not always kept in mind that statements regarding a possible direct influence of hormonal agents are not warranted unless only the particular cell type in question is present in the experimental system This condition holds especially for tissue culture incubations not involving pure cellular types and even for buffy coat preparations in which more than one type is included It is conceivable that the effect of the hormone might be exerted directly upon another cellular line and that the secretory or breakdown products yielded by this element might then exert a secondary action upon the cell examined Experiments should be directed at determining the particular cell type that responds first either on a structural (histochemical) or metabolic basis to the adrenocortical steroids for this finding might provide a clue as to the initial link in the chain of cellular effects induced by these factors Remaining to be determined in this connection is the significance of the lymphopenic reaction preceding chronologically the eosinopenia following administration of pituitary adrenal agents[39]

In summary the evidence remains equivocal as to whether adrenal cortical factors exert a *direct influence upon the lymphocytes in lymphatic organs or in* the body fluids Further experiments are required to test the possibility that other tissues particularly reticuloendothelial in nature mediate the effects of the adrenal cortex and of stress in the destructive process

C *Possible Effects on Redistribution of Lymphocytes*

Indications can be found in the literature that endocrine factors influence the movement of lymphocytic elements from the circulating blood to organs where they may be temporarily sequestered Thus Yoffey[40] has deduced that the

lymph nodes as sites of intravascular and extravascular storage of eosinophils as well as the relation of endocrine factors upon eosinophil migration to and from these tissues

C *Influences upon Eosinophil Destruction*

We have reported elsewhere[67, 68] the existence of degenerating forms of eosinophils in the body fluids of rats following the administration of epinephrine or cortisone or following subjection to the stress of lowered barometric pressures Rat peritoneal fluid which contains relatively large concentrations of eosinophils has been the favored subject of study in these experiments We have demonstrated that a single injection of cortisone produces a drop in the concentration of eosinophils in the peritoneal fluid that is well correlated at six hours and beyond to the eosinopenia occurring in the blood stream of the same animals[67] Degenerative changes are noted clearly in the eosinophil during this time They include chromatolysis karyorrhexis and cytoplasmic fragmentation changes reminiscent of those occurring in the process of lymphocytic destruction[16] Many of the degenerating forms of eosinophils in the peritoneal and pleural fluids are ingested by free mononuclear cells Similar destructive changes in peritoneal eosinophils after treatment with adrenal cortical factors have been reported by Higgins[69]

Degenerating forms of eosinophils are observed in the peripheral blood[68] Their numbers can be augmented significantly in as short a period as 45 minutes following the injection of a single dose of epinephrine an increase that is maintained for at least eight hours Evidence of eosinophil destruction is also to be found within the lymph nodes[70] The peripheral eosinopenia developing after a six hour exposure to lowered barometric pressures is accompanied by a decrease in the percentages of normal eosinophils associated with an elevation in the concentration of both intermediary degenerating eosinophilic forms (eosinorrhexicytes) and anuclear cytoplasmic fragments (eosinomeres) within the ileocecal lymph nodes Romani[66] has observed widespread degeneration of eosinophils in the tissues of animals subjected to certain stresses The suggestion is made[66] that the 11-oxygenated adrenal cortical factors potentiate eosinorrhexis in the lung lymph nodes common connective tissues and other organs in which the destruction phenomenon is observable

It is of interest that signs of eosinorrhexis can be noted in the ileocecal nodes of adrenalectomized animals subjected to low pressures but the intensity of the reaction is not as great as that observed in intact rats exposed to the same stimulus[70] Degenerating eosinophils are also visualized in the peripheral blood of the adrenalectomized rat exposed to anoxia Here however it is accompanied by a significant increase in the numbers of normal eosinophils[71] We are led to postulate that the pituitary adrenal cortical axis functions in at least three ways in regulating the levels of circulating eosinophils (1) it sensitizes the eosinophil within the organism to normally-occurring destructive processes that are independent of the adrenal the reticuloendothelial system aiding in the disposal of the degenerating elements (2) it inhibits the mobilization of eosinophils from sites in which they may be stored or sequestered for transfer

terone and Compound L, under some conditions may invoke lymphoid hypertrophy, the possibility must be considered also that these actions may become unmasked following chronic administration of whole adrenal cortical extracts It would be of interest in this connection to examine the effects on the lymphoid elements of aldosterone, the newly discovered steroid which has been described as possessing properties characteristic of both the 11-oxy and desoxy types of steroids[53, 54]

II Eosinophils

A decrease in the numbers of circulating eosinophils has been repeatedly described as an accompaniment or sequel to the lymphopenia following treatment with ACTH, adrenal factors or application of stress As discussed above in regard to the lymphocyte, several mechanisms suggested themselves in connection with this associated eosinopenic state

A *Effect on the Bone Marrow*

The available evidence does not lend support to the view that the eosinopenia which develops within a few hours following the administration of adrenal cortical principles or of ACTH is due to an inhibition of the production of eosinophils by the bone marrow Thus little change is apparent in the eosinophil pattern of the marrow at the time the peripheral eosinopenia is marked[55, 57] The alterations in eosinophil numbers that do occur are seen after the acute eosinopenic reaction has subsided at which time (8 to 12 hours after cortical hormone administration) increases in the percentages of young eosinophilic elements are noted a compensatory reaction that develops presumably to replace the peripheral eosinophil cells[58] There can be no doubt however that chronic administration of cortical steroids results in a decreased production of eosinophils by the marrow as will be seen below

B *Possible Effects on Distribution of Eosinophils*

The fact is now well established that the spleen may serve as a reservoir for eosinophils that may be discharged rapidly into the circulation following stimulation of the adrenal medullary or cortical components The temporary rise in the levels of circulating eosinophils induced in the intact rat[59] or guinea pig[60] by epinephrine can accordingly be prevented by splenectomy

Some experiments have supported the concept that the eosinopenic reaction associated with pituitary adrenal stimulation originates at least in part from a migration of eosinophils from the circulating blood into organs such as the spleen[61] Not consistent with this contention are the findings that removal of the spleen does not prevent the peripheral eosinopenia following cortisone epinephrine or stress[12, 6, 64] as are the observations that administration of ACTH or epinephrine to dogs does not lead to differences in splenic arteriovenous eosinophil numbers[64] More recent experiments[65, 66] in fact indicate that the 11-oxysteroids tend to inhibit rather than to accelerate the migration of eosinophils into certain tissues Studies are required to determine in more detail the role of organs especially the spleen lungs bone marrow liver and

patient[77] Along the same lines it has been reported that the intravenous administration of epinephrine produces in man an eosinopenia which is not accompanied by any rise in the blood[78] or urinary[77] levels of 17 hydroxycorticosteroids On the other hand the eosinopenia resulting from ACTH injections is almost invariably associated with an increase in the titers of circulating 17 hydroxycorticosteroids[14, 78] The assumption by Spengler[79] that epinephrine results in an increased peripheral utilization of adrenal cortical steroids appears to be rendered untenable by the observations[78] that epinephrine does not affect the rate of disappearance from the blood of exogenously administered cortisone or hydrocortisone It is pertinent to mention that, according to Muehrcke and associates,[80] the ability of epinephrine to induce eosinopenia in the bilaterally adrenalectomized and orchidectomized patient is dependent on the maintenance of a eucortical state through the supplying of small noneosinopenic inducing doses of cortisone In this connection a synergistic action in depressing circulating eosinophil levels has been reported for epinephrine given in conjunction with adrenal cortical factors in the Addisonian subject[81] or bilaterally adrenalectomized patient[83] Speirs[82] has shown that a correlation exists between the eosinopenic response and the presence of adrenal accessories or remnants in adrenalectomized mice treated with epinephrine In fact these and other observations have led Speirs (personal communication) to conclude that if an eosinopenia is produced by epinephrine it is a certain sign of the presence of some functioning adrenal cortical tissue

Thevathasan and I (unpublished) have recently investigated the question relating to the possible synergism of epinephrine and adrenal cortical factors on the eosinophils in the adrenalectomized splenectomized rat utilized at 3 to 4 days after the combined operation The spleen was removed to preclude its possible operation as a reservoir or as a storage depot for eosinophils Different dosages of epinephrine and adrenal cortical extract were utilized but only one set of experiments will be cited in this report As many be seen from FIGURE 2 neither the aqueous beef adrenal cortical extract (Upjohn) given in two 1 0 ml doses over a four hour period nor a 200 μg dose of epinephrine when administered alone resulted in eosinopenia When however both were injected *i.e.* 1 0 ml of cortical extract given first followed four hours later by the 200 μg of epinephrine and another ml of the cortical extract a significant eosinopenia occurred at four hours subsequent to the last injection The 1 ml of 10 per cent ethyl alcohol used as the vehicle for the cortical extract injected alone or in conjunction with the epinephrine at the same time intervals as for the epinephrine-cortical extract treated group gave no significant depression in eosinophil numbers Similarly the 0 5 ml of 1 per cent saline used as the vehicle for the epinephrine when administered separately or together with the cortical extract did not induce eosinopenia

It is difficult to say from these experiments whether (1) the priming with the adrenal extract has allowed a fundamental eosinopenic action of epinephrine to become revealed or (2) whether the epinephrine has potentiated the effect of the adrenal cortical factors A permissive role of the adrenal cortical steroids in epinephrine action[83] upon the blood elements would be an attractive hypothesis especially if evidence for it could be obtained in the adrenal-de

into the circulating blood (3) it inhibits, in the chronic experiment (see below), the production of eosinophils by the bone marrow

The problem has not as yet been clearly resolved as to whether the cortical steroids exert a direct destructive influence upon the eosinophil or induce their eosinopenic action by indirect mechanisms seen only under *in vitro* conditions Evidence of a direct action is derived from the experiments of Muehrcke *et al*[7] who have observed eosinopenia in human defibrinated blood incubated with cortisone Addition of heparin appears to block this *in vitro* action of cortisone On the other hand Esselier and his associates[73] have reported that incubation of citrated, defibrinated, or heparinized blood with cortisone or hydrocortisone for as long a period as 24 hours results in no significant alterations in the numbers of eosinophils or total leukocyte counts of the sample Their conclusion is justified that these findings do not support a direct eoslnorrhectic action of the steroids upon the eosinophil These investigators, however, have interpreted the data of Padawer and Gordon[67, 68] to mean that the presence of degenerative forms of eosinophils following administration of cortical steroids to the organism implies a *direct* destructive action On the contrary, it is our feeling that the steroids act in an indirect manner a contention supported by our inability to observe destructive changes in the eosinophils of peritoneal fluid incubated *in vitro* with cortisone (Padawer and Gordon unpublished) The possibility has arisen that the liver or other organs metabolize the administered steroid to produce intermediary compounds that affect directly the eosinophil and perhaps other formed elements of the blood Speirs (personal communication) however has tested recently a series of steroids obtained by circulating cortisone through the isolated liver preparation and has found them to be ineffective on the eosinophil either within the organism or in the isolated system

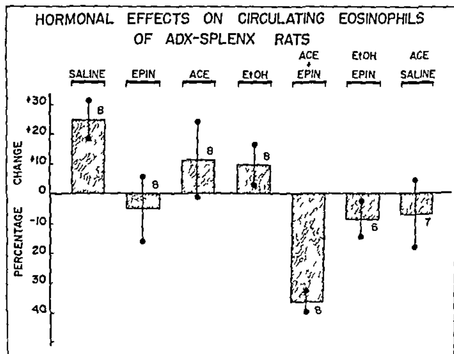
The observation[74] that the implantation of a cortisone pellet directly into the peritoneal cavity of the mouse produces a peripheral eosinopenia before the reduction in eosinophil numbers can be detected in the peritoneal fluid further supports, but does not prove conclusively the contention that the cortical steroids act on the eosinophil through indirect mechanisms That the reticuloendothelial system may constitute a site of action of the cortical steroids is suggested by the work of Esselier and Wagner[75] who have found that blockade' of this system in the guinea pig with trypan blue prevents the eosinopenic reaction to ACTH It remains to be determined whether this effect involves a reduced uptake of the degenerating forms of eosinophils observed in the experiments of Padawer and Gordon[67] Requiring attention and corroboration is the finding that the serum obtained from rats stressed with epinephrine induces an eosinopenia within 30 minutes following its administration[76] It should be recalled that a considerably longer time is required before a significant reduction in eosinophil numbers can be observed following injection of epinephrine or cortisone

The view that the eosinopenia induced by epinephrine or stress is an infallible criterion of a functional pituitary adrenal cortical relation has been challenged recently doubt stemming mainly from the observation that epinephrine may cause eosinopenia in the Addisonian or in the bilaterally adrenalectomized

ing evidence that the neutrophilia induced by stress and pituitary factors may involve, at least in part, extra adrenal mechanisms and may be associated with nonspecific reactions to foreign proteins or other stressful stimuli[19] Thus Palmer *et al*[85] have reported that ACTH will evoke neutrophilia in the adrenal ectomized as well as in the intact rat Since it has been demonstrated[86, 87] that the growth hormone causes a marked rise in neutrophilic granulocytes the possibility of contamination of ACTH preparations with other pituitary factors must be considered in the final evaluation of the results Stein and her co-workers[88] have indicated that epinephrine produces in the intact mouse a more marked peripheral neutrophilia than does adrenal cortical extract The non specificity of this peripheral response is demonstrated in their finding that the neutrophilia can be evoked as well as epinephrine in the adrenalectomized or splenectomized mouse It is conceivable that the adrenal medullary factor operates upon the polymorphonuclear elements through extra adrenal cortical mechanisms involving a specific action on the bone marrow[89, 90] on the release of neutrophils from tissue sites into the blood[91] or through sequestration of cells within parts of the vascular bed itself Also the possibility cannot be ignored that epinephrine alters the concentration in the plasma of a factor reported to cause the expulsion of granulocytes from the marrow into the circulation[92]

There can be no doubt that the adrenal cortical steroids induce alterations in the neutrophilic cell patterns of the bone marrow Thus a single injection to intact mice of a large dose of cortisone results in a significant increase in the marrow myeloid erythroid cell ratio coinciding at some intervals during the nine day period studied with the appearance of peripheral neutrophilic granulocytosis[93] Yoffey and his associates[94] have reported recently that the daily administration for seven days of 11 oxycorticosteroids to intact guinea pigs induces an increase in the cellularity of the marrow involving both the neutrophilic and erythroid elements On the other hand chronic administration of cortisone to adrenalectomized rats results in a significant depression in the per centages and an increase in the relative maturity of the neutrophilic granulocytes of the marrow[95] Similarly recent studies by Fruhman and Gordon[2] have shown a diminution to occur in the total numbers of neutrophilic elements of femoral marrow following treatment for a week with daily 4 mgm doses of Kendall s Compound B (corticosterone) These latter observations lead one to question the validity of the contention that an increased rate of myeloid cellular proliferation within the bone marrow is entirely responsible for the granulocytosis noted in animals treated chronically with steroid factors

In passing it should be remembered that the adrenal cortical steroids and ACTH can exert dramatic although unfortunately only temporary ameliorative effects in the myelogenous leukemias a finding that indicates some as yet im perfectly understood relation between the adrenal cortex and myelopoiesis The cause of the refractoriness that develops in these cases of continued ad ministration of the steroid is a problem that merits concentrated attack A part of the solution may lie in the intermediary metabolism undergone by the steroid molecules during their sojourn in the tissues of the patient afflicted with the blood disease



F URE 2 Influ ce of ep phr e and dre al o t c le tra t g sep t ly a d c mb ati n po the me per t hanges n i cul t g o ph l N mbe s f mals employ d re i d c ted t top of b s V t c l li s th ugh th ba s present ±l ta d d ro of th m n ee t t fo fu th d ta l of e pe imental pro dures G o p 1 0 5 ml 1% sal G o p 2 200 μg p ph 0.5 ml 1% sal ne G p3 1 ml q e s adr al o t c le tra t (ACE) n 10% thyl al h l (EtOH) at 0 h foll w d by a se d 1 ml d f ACE t 4 hr G p 4 1 ml 10% EtOH t 0 h f ll wed by se d 1 ml d se f 10% EtOH at 4 hrs G up 5 1 ml ACE at 0 hr f llow d t 4 hrs by se d 1 ml d se f ACE n jun t w th 200 μg p ph ne 0 5 ml 1 sal n G up 6 1 ml 10% EtOH at 0 h f llow d at 4 h by a se ond 1 ml d se f 10% EtOH g n i j ct o w th 200 g p ph i 0 5 ml 1% s l G o p 7 1 ml ACE at 0 hr f llow d t 4 hrs by se o d 1 ml d se f ACE g en n jun t n w th 0 5 ml 1% sal e

medullated sympathectomized organism If the second hypothesis should prove correct it would be desirable to have a more complete knowledge of the mechanisms by which epinephrine and other stressor agents condition or alter the sensitivity of the tissues including the blood elements to adrenal cortical factors A clue to this problem may well be provided in Selye s[19] suggestion that such conditioning action is related to the catabolic phases of the alarm reaction, a time when the acute effects of stress upon the blood elements are best visualized

III Neutrophils

As has been noted above a neutrophilia is coexistent with the lymphopenia and eosinopenia following administration of pituitary adrenal factors or sub jection to stressful stimuli Duty[34] observed that a negative correlation exists between the numbers of circulating neutrophils and the adrenal ascorbic acid content in the rat and concluded that the adrenal cortex plays a role in the regu lation of the numbers of circulating neutrophils Nevertheless there is increas

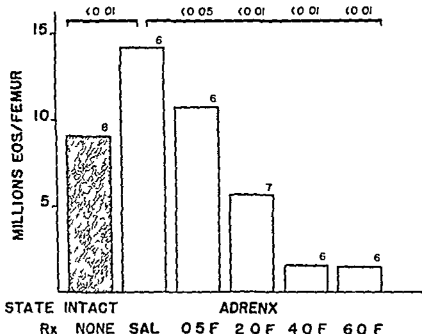


FIGURE 3 Eff t f a ious dosage f hydroc rt so po th m an tot l umber of e ph l with th fem ral m rrow f dre l t m zed t Numbe f a mal employed re d t d t t p f b rs p l l lated f om th d t b t on l} h t re h w a ros th t p f the gr ph M a l f th trol dre l tom zed gr p (SAL, ba 2) i compared t that f th pe t d t l (INTACT ba 1) M f all g o p j t d th Compo d F (bar 3 t 6) re ompared t th t f th sal j t d ad e le tom zed t (b 2) Se te t f d t l f pe me tal procedu

eosinophil numbers are increased after adrenalectomy and decreased proportionately with increasing doses of Compound F Adrenal removal also results in an increase in the total numbers of marrow lymphocytes with 2 to 6-mgm dosages of Compound F acting similarly in producing a significant reduction in the absolute numbers of lymphocytes (FIGURE 4) Accompanying these changes in eosinophil and lymphocyte numbers are oppositely-occurring alterations in the numbers of nucleated erythroid cells (FIGURE 5) Thus at five days after adrenalectomy there is approximately a 50 per cent reduction in the absolute numbers of nucleated red cells in the femoral marrow Daily dosages as small as 0 5 mgm Compound F (which permit body weight gains in these adrenalectomized rats) produce more than a doubling and 2 to 6 mgm quantities (catabolic doses) result in approximately a 300 per cent increase in the total numbers of nucleated erythrocytes Not indicated in the figures is the fact that adrenalectomy is followed by a significant reduction in the total numbers of mitoses encountered among the nucleated erythroid elements increasing dosages of Compound F producing a marked increase in their numbers within the femoral marrow Neutrophilic cell numbers show a trend towards a decrease with the 4 and 6-mgm amounts of Compound F

Hayes and Baker[16] have reported that chronic treatment of intact rats with

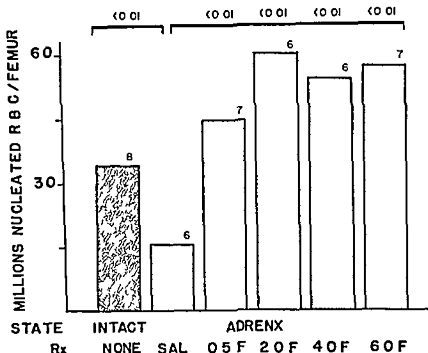
IV Quantitative Studies of Bone Marrow

Our laboratory has been engaged during the past few years in a study of the influence of endocrine deficiencies and of some forms of replacement treatment upon bone marrow structure and metabolism The method of marrow study employed is a quantitative one devised by Fruhman[22] which permits the determination of the total numbers of the various cell types in the bone marrow of the rat A brief description of the method follows

At the time of sacrifice, the animals are anesthetized lightly with ether and exsanguinated by cardiac puncture The femurs are dissected quickly trimmed completely of all surrounding tissue, cracked open carefully, and weighed before and after removal of the marrow The remaining fragments of marrow are removed by swabbing the interior walls of the bone with small squares of lens paper *If the determinations are conducted properly, the marrow weights* of the right and left femurs seldom differ by more than 5 per cent The rat femur is especially desirable for a study of this type since it is generally devoid of internal spicules of bone It is obviously imperative that no fragments of bone be lost in the procedure lest the weight measurements be rendered inaccurate

A sample of the marrow is drawn into a calibrated 20 cu mm pipette, and the mass of the marrow is determined by weighing the pipette with and without the marrow sample With the weight of a given volume of marrow known a calculation now provides the bone marrow density The marrow is then expelled into a tube containing exactly 2 ml of homologous serum, and a uniform suspension is formed by drawing the marrow up and down in a pipette Samples are now taken for hemacytometer counts and for smears One sample is diluted with Tyrode s solution and the second with Randolph s staining fluid Having calculated the total femoral marrow volume the first count allows the determination of the total numbers of cells within the femur With the aid of the second count the total numbers of nucleated cells and of eosinophils are ascertained directly The difference between the two counts represents the numbers of nonnucleated red blood cells in the sample By employing the myelogram cell percentages from stained smears it is possible to calculate the absolute numbers of the other cellular types This method provides a rapid reproducible means for estimating the total cellular numbers in rat bone marrow

In FIGURES 3 to 5 are shown the effects of adrenalectomy and of several dosages of Compound F (17 hydroxycorticosterone) upon the total numbers of some cellular types of femoral bone marrow of the rat Six groups of adult male rats were established Group 1 comprised the intact untreated controls Croups 2 to 6 consisted of the adrenalectomized animals The animals of Croups 2 to 6 were primed daily for two days before adrenalectomy with subcutaneous injections of from 0 5 to 6 0 mgm of Compound F or with 1 0 ml of 1 per cent saline the vehicle for the steroids (Group 2 SAL) Adrenalectomy was performed on the third day and injections continued on this day and daily for the subsequent four days All rats were killed on the eighth day five days after adrenalectomy It will be noted from FIGURE 3 that total



F G R 5 Eff t f d sage f hyd oc t so pon th mean t tal mbe f l ted ryth cyt w th the f m l m ow f ad l tom zed t N mbe f an m l mpl y d d cat d t t p f b P l l lat d f m th d t b t f F h t hown th t p f th gr ph M a l f th t l d l tom zed g up (SAL b 2) mp red t th t f th pe t d t l (IN TACT b t) M f ll groups j ted w th Compo d F (b 3 t 6) e mpa d t th t f th sal j ted adre l tomized ts (b 2) See te t f d t l f pe m tal p oc d

ment treatment with various doses of Compounds B and F exerted little influence upon the oxygen uptake of marrow.[71] It was noted, however, that adrenalectomy resulted in approximately a 2- per cent increase ($P < 0.001$) in the rate of marrow anaerobic glycolysis using glucose as the substrate. In the present studies the effects of Compound B administered as a daily 2 mgm dose and Compound F in four different dosages were observed upon the anaerobic glycolysis of adrenalectomized rat bone marrow. The design of the experiment was identical to that utilized in the marrow volume studies of Fruhman and Gordon.[23] In fact, marrow samples for the manometric studies in the control adrenalectomized and Compound F injected groups were obtained from the same animals employed in the marrow volume studies described above.

FIGURE 6 illustrates the high rate of anaerobic glycolysis characteristic of adrenalectomized rat bone marrow. Of especial significance is the fact that increasing dosages of Compound F result in proportionate reductions of glycolytic activity. Also indicated is the group of adrenalectomized rats treated with Compound B which is seen to be as effective as Compound F in depressing the glycolysis of bone marrow. Inhibitory effects of the 11 oxysteroids have been observed under *in vitro* conditions for rodent lymphatic cells and mouse

MILLIONS LYMPHOCYTES/FEMUR

< 0 05 < 0 30 < 0 01 < 0 01 < 0 01

8 6 6 7 6 6

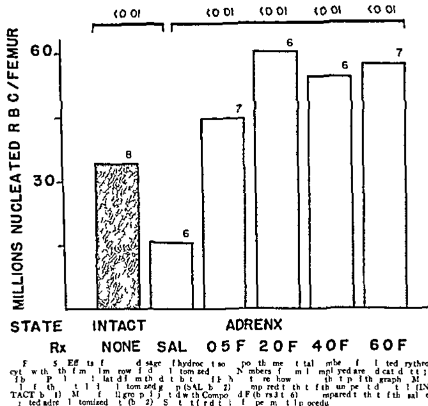
STATE INTACT ADRENX

Rx NONE SAL 0 5 F 2 0 F 4 0 F 6 0 F

Fr ur 4 Effe ts f var u d ages of hyd c t so pon the m a t tal number of lymphocyte w th n th f moral mar ow of d n l tom zed ts Numbe s f a mal employ d re i d cated at top f b rs P al al lated f om th d tr but o f F he t a e show s th t p of th gr ph M a al for th co tr l ad l ct m zed g oup (SAL ba 2) s ompar d to th t f the u pe t d t ols (INTACT bar 1) M an for ll g o ps i j ct d w th Compou d F (ba s 3 to 6) ar mp red t that of the sal e je t d dre al t m zed ats (b 2) Se t xt f r d ta ls f pe mental proc d r s

an adrenal cortical extract (lipoadrenal cortex) results in an increased cellularity of the bone marrow associated with a marked increase in the percentages of nucleated erythroid cells and with reductions in the percentages of eosinophils and neutrophils within the marrow Yoffey *et al* [94] detected no alterations in the concentration (numbers per cu mm of marrow) of lymphocytes and eosinophils in the bone marrow of intact guinea pigs treated daily for seven days with 11 oxysteroids As already mentioned these workers also reported that Compounds E and F produced an increased cellularity of the marrow involving the myeloid and especially the erythroid elements the latter point expressing agreement with the experiments of Fruhman and Gordon [22] The discrepancies noted in Yoffey s work and ours may be traced most likely to the differences in species employed the guinea pig being more refractory than the rat to adrenal cortical steroid treatment In addition the animal deprived of its adrenals appears in our experience to be a more sensitive test object than the intact animal for a study of the influence of adrenal factors upon the blood elements

Dornfest and Gordon[100] have examined the respiratory and glycolytic activity of pooled femoral and tibial bone marrow suspensions obtained from rats that were adrenalectomized and given various forms of replacement treatment Standard manometric procedures were employed The reader is referred to Gordon[71] for the details of the techniques Adrenalectomy as well as replace



F S Eff ts f d sage f hydroc t so po th me t tal mbe f l ted rythro cyt w th th f m lm row f d l tom zed N mbers f m l mpl yed are d cat d t t j f b P l l lat d f m th d t b t f F h t re how th t p f th graph M l f th t l l l tom zed g p (SAL b 2) mp red t th t f th un pe t d t l (INTACT b l) M f ll gro p i j t d w th Compo d F (b rs 3 t 6) mpared t th t f th sal e j ted adre l tomized t (b 2) S t t f r d t l f pe m t l p ocedu

*ment treatment with various doses of Compounds B and F exerted little in*fluence upon the oxygen uptake of marrow[71] It was noted however that adrenalectomy resulted in approximately a 25 per cent increase ($P < 0.001$) in the rate of marrow anaerobic glycolysis using glucose as the substrate In the present studies the effects of Compound B administered as a daily 2 mgm dose and Compound F in four different dosages were observed upon the anaerobic glycolysis of adrenalectomized rat bone marrow The design of the experiment was identical to that utilized in the marrow volume studies of Fruhman and Gordon[22] In fact marrow samples for the manometric studies in the control adrenalectomized and Compound F injected groups were obtained from the same animals employed in the marrow volume studies described above

FIGURE 6 illustrates the high rate of anaerobic glycolysis characteristic of adrenalectomized rat bone marrow Of especial significance is the fact that increasing dosages of Compound F result in proportionate reductions of glycolytic activity Also indicated is the group of adrenalectomized rats treated with Compound B which is seen to be as effective as Compound F in depressing the glycolysis of bone marrow Inhibitory effects of the 11 oxysteroids have been observed under *in vitro* conditions for rodent lymphatic cells and mouse

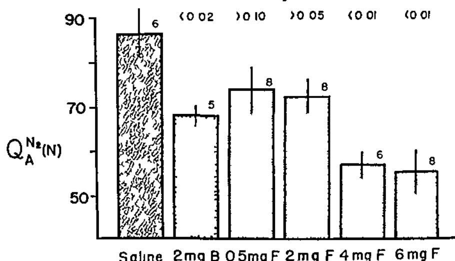


FIGURE 6 Eff t f rtc ter e d v u d sage of hyd o t s p n m a aer bic gly lyt l s (μl CO_2/mg N/h) f r d l t miz d at bo m rr w N mbe f m l empl yed re i d t d t t p f b r V rti al li th o gh th ba s r p ese t ±1 t d d of th m P l re sh w a th t p of th gr ph Means for all h rm t e t d d l t m zed g oups (ba s 2 to 6) a mp d to that of th l t ted c t l dr al t m zed g o p (ba 1)

diaphragm[97, 98] and also for human buffy coat leukocytes [99] The observations that the corticosteroids inhibit hexokinase activity, and that the addition of glutathione will protect the isolated mouse diaphragm from the inhibitory action of Compound F on glycolysis has led to the conclusion that the adrenal cortical hormones inhibit glycolysis by combining with the —SH groups of hexokinase [98]

It would be of interest to ascertain, as closely as possible with the available techniques the time sequence of development of the glycolytic and morphologic alterations observed in the marrow following adrenal removal This finding might provide an answer to the basic question as to whether a change in metabolism is the cause of the result of or simply an accompaniment to the structural alterations seen in the adrenal insufficient and adrenal steroid injected animal

V Conclusions

It would seem from the evidence reviewed in this report that the endocrine system plays a role in the regulation of the numbers of leukocytes within the circulation through influences exerted upon their production by the blood forming organs their distribution in the tissues and their eventual disposal Further experiments should consider in greater detail the mechanisms underlying the action of hormones upon hemopoietic and blood destroying processes In this connection examination should be made of the modifications produced by endocrine deficiencies and by hormonal replacement and overdosage upon the marrow enzyme systems especially the peptidases and esterases which have thus far been largely neglected Phosphorus iron copper and cobalt turnover as well as various substrate utilization studies should also prove rewarding

There is need for further application of quantitative procedures such as those described in the body of this report in the evaluation of hormonal influences upon the absolute numbers of blood elements within the blood forming organs Finally attempts should be made to ascertain the precise relation of the metabolic to the morphologic changes induced by endocrine manipulation in the leukocytes within the body fluids and hemopoietic tissues The separation of the various leukocytic types already partially accomplished in our laboratories should prove a significant step in this direction

Acknowledgments

The author wishes to thank Doctor W J Haines of the Upjohn Company *for generous supplies of aqueous adrenal cortical extracts and Compounds* B and F used in the original experiments He is also indebted to Doctor J Padawer and to Myron Tannenbaum for help rendered in preparing the illustrations

Bibliography

1 Addison T 1855 On the Constitutional and Local Effects of Disease of the Supra Renal Capsules Samuel Highley London England
2 Silver S 1933 Arch Internal Med 51 175
3 Kocher T 1908 Arch klin Chir 87 131
4 Zwemer R L & C Lyons 1928 Am J Physiol 86 545
5 Corey E L & S W Britton 1932 Am J Physiol 102 699
6 Shecket, H A D L Friedman & L D Nice 1935 Proc Soc Exptl Biol Med 32 608
7 Dalton A J & G Maslon 1940 Proc Soc Exptl Biol Med 43 370
8 Selye H 1937 Endocrinology 21 169
9 Dalton A J & H Selye 1939 Folia Haematol 62 397
10 Dougherty T F & A White 1944 Endocrinology 35 1
11 Hills A G P H Forsham & C A Finch 1948 Blood 3 755
12 Forsham P H G W Thorn F T Prunty & A G Hills 1948 J Clin Endocrinol 8 15
13 Speirs R S & R K Meyer 1951 Endocrinology 48 316
14 Speirs R S 1953 Endocrinology 52 300
15 Nelson D H 1953 Ciba Foundation Colloquia on Endocrinology 5 162
16 Dougherty T F 1952 Physiol Revs 32 379
17 Stoerk H C 1946 Proc Soc Exptl. Biol Med 62 90
18 Nakashima R., H C Stoerk & A S Gordon Unpublished
19 Selye H 1950 Stress Acta Inc Montreal
20 Santisteban G A & T F Dougherty 1954 Endocrinology 54 130
21 Money W L., J Fager & R W Rawson 1952 Cancer Research 12 206
22 Fruhman G J & A S Gordon Unpublished
23 Baker B L D J Ingle & C H Li 1951 Am J Anat 88 313
24 Andreasen E & S Christensen 1949 Anat Record 103 401
25 Michael S T 1949 Yale J Biol and Med 22 71
26 Dougherty T F & L F Kumagai 1951 Endocrinology 48 691
27 Heilman D H 1945 Proc Staff Meetings Mayo Clinic 20 310
28 Holden M B C Seegal & I Ryby 1951 Am J Path 27 748
29 Baldridge G D A M Kligman M J Lipnik & D M Pillsbury 1951 Arch Path (Lab Med) 51 593
30 Robertson J S 1948 Nature 161 814
31 Delaunay A M Delaunay & J Lebrun 1949 Ann inst Pasteur 76 203
32 Schrek R 1949 Endocrinology 45 317
33 Schrek R 1951 Proc Soc Exptl Biol Med 76 557
34 Hechter O & S Johnson 1949 Endocrinology 45 351
35 Feldman J D 1950 Endocrinology 46 552
36 Feldman J D 1952 Endocrinology 51 258

37 FRANK J A & T F DOUGHERTY 1953 Proc Soc Exptl Biol Med 82 17
38 TROWELL O A 1953 J Physiol 119 274
39 JAKOBSON T & H HORTLING 1954 Acta Endocrinol 15 368
40 YOFFEY J M 1950 Biol Revs Cambridge Phil Soc 25 314
41 FARR R S 1951 Anat Record 109 515
42 YOFFEY J M W K METCALF G HERDAN & V NAIRN 1951 Brit Med J 1 660
43 HUDSON G G HERDAN & J M YOFFEY 1952 Brit Med J 1 999
44 HECHTER O 1948 Endocrinology 42 285
45 STONE D & O HECHTER 1948 Endocrinology 42 307
46 WEISBERGER A S R A GUYTON R W HEINLE & J P STORAASLI 1951 Blood 6 916
47 REINHARDT W O & C H LI 1945 Science 101 360
48 YOFFEY J M M REISS & J S BAXTER 1946 Nature 157 368
49 HUNGERFORD G F W O REINHARDT & C H LI 1952 Blood 2 193
50 YOFFEY J M & J S BAXTER 1946 J Anat 80 132
51 INGLE D J 1949 Ann N Y Acad Sci 50 576
52 ENGEL F L 1953 Protein Metabolism Hormones and Growth 34 Rutgers Univ Press New Brunswick N J
53 SPEIRS R S S A SIMPSON & J F TAIT 1954 Endocrinology 55 233
54 GAUNT R A S GORDON A RENZI J PADAWER G J FRUHMAN & M GILMAN 1954 Endocrinology 55 236
55 GODLOWSKI Z Z 1948 Brit Med J 1 46
56 BEST W R & M SAMTER 1951 Blood 6 61
57 *ASCHKENASY A 1952 Semaine Hôp Paris 28 1*
58 DURGIN M L & R K MEYER 1951 Endocrinology 48 518
59 McDERMOTT W V E FRY J R BROBECK & C N H LONG 1950 Yale J Biol and Med 23 52
60 SCHWEIZER M 1953 Endocrinology 53 293
61 SPAIN D M & W THALHIMER 1951 Proc Soc Exptl Biol Med 76 320
62 SPEIRS R S & R K MEYER 1949 Endocrinology 45 403
63 DURY A 1950 Am J Physiol 160 75
64 PFEIFFER E F J SCHUTZ & K SCHOFFLING 1952 Z ges exptl Med 119 347
64a SOLOMON D H & S R HUMPHREYS 1951 Blood 6 824
65 ROMANI J D 1952 Compt rend soc biol 146 1565
66 ROMANI J D 1952 Compt rend soc biol 146 1680
67 PADAWER J & A S GORDON 1952 Endocrinology 51 52
68 PADAWER J & A S GORDON 1952 Proc Soc Exptl Biol Med 80 581
69 HIGGINS G M 1953 Am J Clin Path 23 775
70 FRUHMAN G J & A S GORDON 1953 Proc Soc Exptl Biol Med 82 162
71 GORDON A S 1954 Recent Progress in Hormone Research 10 339 G Pincus Ed Academic Press New York N Y
72 MUEHRCKE R C J L LEWIS & R M KARK 1952 Science 115 377
73 ESSELIER A F P JEANNERET E KOPP & L MORANDI 1954 Endocrinology 54 477
74 PANZENHAGEN H & R S SPEIRS 1953 Blood 8 536
75 ESSELIER A F & K F WAGNER 1952 Klin Wochschr 30 705
76 TANOS B S SZILASY V VARRO A EISNER & F OLAH 1953 Experientia 9 266
77 THORN G W D JENKINS & J C LAIDLAW 1953 Recent Progress in Hormone Research 8 171 G Pincus Ed Academic Press New York N Y
78 SANDBERG A A D H NELSON J G PALMER L T SAMUELS & F H TYLER 1953 J Clin Endocrinol 13 629
79 SPENGLER M 1953 Acta Endocrinol 13 306
80 MUEHRCKE R C T W STAPLE & R M KARK 1952 J Lab Clin Med 40 169
81 RECANT L D M HUME P H FORSHAM & G W THORN 1950 J Clin Endocrinol 10 187
82 SPEIRS R S 1953 Am J Physiol 172 520
83 HENRY W L L OLINER & E R RAMEY 1953 Am J Physiol 174 455
84 DURY A 1948 Endocrinology 43 336
85 PALMER J G G E CARTWRIGHT & M M WINTROBE 1951 Proc 2nd Clinical ACTH Conference 1 438 J R Mote Ed Blakiston Philadelphia Pa
86 CAMPBELL J H R HAUSLER J S MUNROE & I W F DAVIDSON 1953 Endocrinology 53 134
87 CAMPBELL J J S MUNROE H R HAUSLER & I W F DAVIDSON 1953 Endocrinology 53 549

88 Stein K F C Martin & D Falk 1951 Proc Soc Exptl Biol Med 76 110
89 Davis J E 1941 Am J Physiol 134 219
90 Davis J E 194? Am J Physiol 137 699
91 Hamilton L H & S M Horvath 1954 Am J Physiol 176 311
92 Steinberg B & R A Martin 1950 Am J Physiol 161 14
93 Quittner H N Ward L N Sussman & W Antopol 1951 Blood 6 513
94 Yoffey J M R J Ancill J A G Holt B Owensmith & G Herdan 1954 J Anat 88 115
95 Gordon A S S J Piliero & D Landau 1951 Endocrinology 49 497
96 Hayes M A & B L Baker 1952 Univ Mich Med Bull 18 109
97 Kit S & E S G Barron 1953 Endocrinology 52 1
98 Bacila M & E S G Barron 1954 Endocrinology 54 591
99 Martin S J S N Chaudhuri J Green & G R McKinney 1954 J Clin Invest 33 358
100 Dornfest B S & A S Gordon Unpublished experiments

THE QUANTITATIVE STUDY OF THE LEUKOCYTES*

By J M Yoffey

Department of Anatomy The University of Bristol Bristol England

In discussing the various factors which may influence the numbers, distribution, and fate of the leukocytes, it is clear that there are still numerous gaps in our knowledge It would appear probable that an increased functional demand for leukocytes would involve increased production, and this increase, in turn, would require a knowledge of what the functions of the leukocytes really are In the case of the neutrophile granulocytes where an antibacterial action can readily be observed, the classical leukocytosis of many bacterial infections seems a reasonable occurrence In the case of the lymphocytes where an antibacterial action is not obvious, we are not quite so confident in our interpretation of numerical changes When one surveys the general leukocyte field, and the history of our knowledge of that field two significant features emerge The first is that, on the whole, the emphasis has been placed on pathological changes in frank disease conditions notably the infections and blood dyscrasias, and the second is that for the most part the leukocytes have been studied quantitatively only while circulating in the blood

In recent years, it is true attention has been directed to changes in leukocytes in response to what might be considered normal physiological stimuli The present and earlier papers by Gordon and his collaborators (Gordon and Charipper, 1947 Gordon Piliero and Landau 1949 Feigin and Gordon, 1950, Gordon and Katsh 1949) are an excellent example of this tendency, as also are the contributions by Dougherty and his group (Dougherty and White 1944 Dougherty and White 1947 Dougherty and Kumagai 1951 Santisteban and Dougherty, 1954) Crafts (1941, 1948) and others The observations of Richter also represent an interesting development in the study of normal leukocyte functions in the healthy organism irrespective of their role in disease It may well be that, in some instances the function of leukocytes in disease is merely an extension of their function in health On the other hand it is possible that there may be marked differences in the two types of function

In all functional studies however an essential item of information is a quantitative one and is concerned with the numerical aspect of the problem From this point of view the number of leukocytes circulating in the blood stream presents only one facet of the problem historically the first to which serious attention was directed

The blood leukocytes however whether because they are destroyed while in the circulation or because they leave it constitute a shifting cell population and they represent an equilibrium between two processes namely the new formation of leukocytes to be discharged into the blood and the destruction or migration from the blood of the cells already in it For an understanding of the blood leukocytes it becomes essential therefore to know something of the two

* A co s d bl p t of th w k d sc bed th p pe h b n p f rm d w th the d f g a gr t f m M rs Re k tt & Colma Ltd H ll Engl d

processes governing the basic equilibrium For several reasons, the problem of the lymphocytes differs considerably from that of the granulocytes

The Quantitative Study of the Lymphocytes

Though there may be differences of opinion about the so-called ' germinal centers of lymphoid tissue it is generally accepted that one of the main functions of lymphoid tissue in health is the production of lymphocytes Some of these cells may enter the blood by passing through the walls of the blood capillaries present in lymphoid tissue (*e g*, Schulze 1925), while others first enter the lymph stream In the case of the lymph borne lymphocytes cannulation of the thoracic and right lymph ducts enables us to collect these cells immediately before they enter the blood stream The earlier literature dealing with this subject has been reviewed elsewhere (Drinker and Yoffey 1941 Yoffey 1950) and with certain qualifications the results of the various observers appear to be fairly consistent

In counting these lymph borne lymphocytes an obvious question concerns the numbers of those other lymphocytes which are thought to enter the blood stream directly We have no simple way of measuring these but their numbers cannot be great for from the experiments of Blalock *et al* (1937) it would appear that if all the lymphatics of the body are blocked so that no lymph borne lymphocytes can get to the blood stream then the blood lymphocytes fall practically to zero

A further question concerns the numbers of lymphocytes which are daily entering the blood Counts in the dog (Yoffey 1935), cat (Adams *et al*, 1945), and rat (Reinhardt 1945) to mention but a few give a number sufficient to replace those already in the blood stream about twice daily though Sanders *et al* (1940) working with cat and rabbit gave the blood lymphocytes a somewhat higher rate of replacement ranging from three to five times daily

The statement that the blood lymphocytes are replaced twice or more daily is based on the assumption that the lymphocytes entering the blood via the main lymph ducts are in fact newly formed cells It has been argued (*e g* Sjovall 1936) that this assumption is not true and that there is a continuous circulation of lymphocytes between blood and lymph However experiments on the cell content of peripheral lymph which has not yet traversed lymph nodes show that lymph when it is formed contains very few cells (Yoffey and Drinker 1939 Allen 1945) But despite this fact and despite the fact that lymphoid tissue shows such obvious signs of cell multiplication (*cf* Ehrich Drabkin and Forman 1949) it has been argued (Fichtelius 1953) that a circulation of lymphocytes does occur and that the lymphoid tissues are the main site of cell exchange between lymph and blood Experiments such as those of Mann and Higgins (1950) or of Glenn *et al* (1949) in which the escape of lymph through a fistula for several days is associated with a gradual fall in the cell content of lymph are not conclusive for though they could fit in with the concept of lymphocyte circulation they do introduce abnormal factors also

On the whole then the evidence is against circulation of lymphocytes and there still remains the classical problem of the fate of the blood lymphocytes

which are not destroyed in the circulation The lymphocytes must obviously leave the blood and of all the situations suggested, the three which are the most plausible are (1) the bone marrow (2) the lumen of the intestine and (3) the connective tissues all over the body According to Kindred (1942) the lymphocytes in the intestinal wall are about three times as numerous as those present in the blood, and from a numerical point of view, they could account for the missing lymphocytes However the direct evidence for the intestinal elimination of lymphocytes is not convincing As far as the connective tissues are concerned no quantitative data are available concerning their lymphocyte population No investigator has yet undertaken the heroic task of counting the connective tissue lymphocytes in serial sections of an entire animal, or even of trying to obtain an approximate idea of the magnitude of the lymphocyte population by thus examining even a limited mass of connective tissue

A Quantitative Technique for the Estimation of Marrow Cells

The bone marrow as the destination of the blood lymphocytes raises one of the most violent and fundamental of all hematological controversies and before becoming involved in this dispute it seemed worthwhile to ascertain what numbers of lymphocytes the marrow actually contained

The earlier efforts were somewhat crude (Yoffey and Parnell 1944) but, after a number of trials a reasonably satisfactory quantitative technique was finally developed (Yoffey *et al* 1954) based upon earlier work of Gordon (1939) Our standard animal is the young male guinea pig weighing about 400 gm The disadvantage of confining work to a single species is obvious and it is hoped before long to investigate the bone marrow of other species For the time being however we felt it would be more valuable to study intensively in a single species the responses of the bone marrow to a variety of stimuli

A small glass tube internal diameter about ¼ inch and length about 1⅜ inches is provided with a tightly fitting rubber stopper which leaves no recesses between it and the side of the tube The stoppered tube is weighed Under ether anesthesia the abdomen of a guinea pig is opened the aorta is incised and the blood collected in centrifuge tubes which are centrifuged for seven minutes at 3 500 rpm after which the supernatant serum is withdrawn The stoppered tube is about half filled with serum and weighed again Meanwhile a humerus is removed and cleaned and the ends are sawn off with a fine fretsaw By means of a rubber tube and blower attached to one end of the humerus the marrow is ejected into the stoppered tube which is weighed a third time One thus has a known weight of marrow in a known weight of serum

The tube is then placed in a mechanical shaker with an amplitude of about five inches and is shaken for two to three minutes at 400 times per minute The marrow from the guinea pig humerus of this size rapidly disintegrates and produces a uniform cell suspension from which counts can be made in the usual way with a hemocytometer and smears also may be made for a differential count Where the disintegration of the marrow is inadequate and the suspension contains obvious large clumps of cells the count must be discarded Since the suspension contains a number of large cells which sediment rapidly

the tube is shaken each time before taking a count or preparing a smear After the count and smears have been obtained the specific gravity of the suspension and of the serum are measured by the copper sulphate method and the volumes of bone marrow and serum can then be calculated A true volume dilution of marrow in serum is thus obtained and counts are finally expressed as absolute numbers of cells per cu mm of marrow

The differential counts can be made either in supravital preparations or dry smears We have preferred the dry smears stained usually with MacNeal s tetrachrome stain because they yielded much more nuclear detail However they have the defect of containing a fair number of damaged cells about whose identity we are not altogether sure (Yoffey *et al* 1954) The elusive reticulum cells may contribute some of the damaged cells as the granulocytes also undoubtedly do Supravital preparations contain far fewer damaged cells but in our hands possibly because we have not had long experience with the supravital technique they have yielded less satisfactory results

Another problem in the making of dry smears concerns the cell distribution If the smears (slide method) are made rapidly the distribution of the cells is uniform but the individual cells are rounded and not spread out so that identification is impossible If the smears are made slowly individual cells are spread out and show up much better but there are many more damaged cells and the distribution is no longer uniform since the larger cells accumulate at the edge of the smear In each experiment therefore 15 to 20 smears are made at different speeds and finally one or two of the best ones selected The presence of the damaged cells will not of course affect the accuracy of the absolute counts of the cells which can be identified but if the various cell groups are not uniformly damaged their ratios to one another may be altered Because of their small content of damaged cells it may yet prove that supravital preparations will be the method of choice

Exsanguination of the animal by cutting the abdominal aorta is important not merely to obtain autogenous serum but also in order to drain away from the bone marrow as much blood as possible If the animal is not exsanguinated the amount of residual blood in the vessels may be quite appreciable though some information about it may be obtained by performing both a red cell count and a reticulocyte count on the marrow suspension It is probable that the vast majority of the marrow reticulocytes are red cells which have just been formed in the marrow and are about to be discharged into the blood

Number of Lymphocytes in Bone Marrow

As far as the total number of nucleated cells in the normal marrow is concerned we find they number in the neighbourhood of 1,500 000 per cu mm (1 235 000 Yoffey Metcalf Herdan and Nairn 1951 1 248 000 Hudson Herdan and Yoffey 1952 1 690 000 Yoffey Ancill Holt Owen Smith and Herdan 1954 1 393 000 Harris Ancill Herdan and Yoffey 1954) The absolute figures for the lymphocytes in these four groups of experiments were respectively 145 000 246 000 322 000 and 314 000 per cu mm of marrow

The lymphocytes are virtually all small lymphocytes and the quantitative data make it clear beyond all doubt that they are present in bone marrow in

which are not destroyed in the circulation The lymphocytes must obviously leave the blood and, of all the situations suggested the three which are the most plausible are (1) the bone marrow (2) the lumen of the intestine, and (3) the connective tissues all over the body According to Kindred (1942), the lymphocytes in the intestinal wall are about three times as numerous as those present in the blood and, from a numerical point of view they could account for the missing lymphocytes However, the direct evidence for the intestinal elimination of lymphocytes is not convincing As far as the connective tissues are concerned no quantitative data are available concerning their lymphocyte population No investigator has yet undertaken the heroic task of counting the connective tissue lymphocytes in serial sections of an entire animal or even of trying to obtain an approximate idea of the magnitude of the lymphocyte population by thus examining even a limited mass of connective tissue

A Quantitative Technique for the Estimation of Marrow Cells

The bone marrow as the destination of the blood lymphocytes raises one of the most violent and fundamental of all hematological controversies and before becoming involved in this dispute it seemed worthwhile to ascertain what numbers of lymphocytes the marrow actually contained

The earlier efforts were somewhat crude (Yoffey and Parnell 1944) but, after a number of trials a reasonably satisfactory quantitative technique was finally developed (Yoffey *et al* 1954) based upon earlier work of Gordon (1939) Our standard animal is the young male guinea pig weighing about 400 gm The disadvantage of confining work to a single species is obvious and it is hoped before long to investigate the bone marrow of other species For the time being however we felt it would be more valuable to study intensively in a single species the responses of the bone marrow to a variety of stimuli

A small glass tube internal diameter about ¼ inch and length about 1⅜ inches is provided with a tightly fitting rubber stopper which leaves no recesses between it and the side of the tube The stoppered tube is weighed Under ether anesthesia the abdomen of a guinea pig is opened the aorta is incised and the blood collected in centrifuge tubes which are centrifuged for seven minutes at 3 500 rpm after which the supernatant serum is withdrawn The stoppered tube is about half filled with serum and weighed again Meanwhile a humerus is removed and cleaned and the ends are sawn off with a fine fretsaw By means of a rubber tube and blower attached to one end of the humerus the marrow is ejected into the stoppered tube which is weighed a third time One thus has a known weight of marrow in a known weight of serum

The tube is then placed in a mechanical shaker with an amplitude of about five inches and is shaken for two to three minutes at 400 times per minute The marrow from the guinea pig humerus of this size rapidly disintegrates and produces a uniform cell suspension from which counts can be made in the usual way with a hemocytometer and smears also may be made for a differential count Where the disintegration of the marrow is inadequate and the suspension contains obvious large clumps of cells the count must be discarded Since the suspension contains a number of large cells which sediment rapidly

the tube is shaken each time before taking a count or preparing a smear After the count and smears have been obtained the specific gravity of the suspension and of the serum are measured by the copper sulphate method and the volumes of bone marrow and serum can then be calculated A true volume dilution of marrow in serum is thus obtained and counts are finally expressed as absolute numbers of cells per cu mm of marrow

The differential counts can be made either in supravital preparations or dry smears We have preferred the dry smears stained usually with MacNeal s tetrachrome stain because they yielded much more nuclear detail However they have the defect of containing a fair number of damaged cells about whose identity we are not altogether sure (Yoffey *et al* 1954) The elusive reticulum cells may contribute some of the damaged cells as the granulocytes also undoubtedly do Supravital preparations contain far fewer damaged cells but in our hands possibly because we have not had long experience with the supravital technique they have yielded less satisfactory results

Another problem in the making of dry smears concerns the cell distribution If the smears (slide method) are made rapidly the distribution of the cells is uniform but the individual cells are rounded and not spread out so that identification is impossible If the smears are made slowly individual cells are spread out and show up much better but there are many more damaged cells and the distribution is no longer uniform since the larger cells accumulate at the edge of the smear In each experiment therefore 15 to 20 smears are made at different speeds and finally one or two of the best ones selected The presence of the damaged cells will not of course affect the accuracy of the absolute counts of the cells which can be identified but if the various cell groups are not uniformly damaged their ratios to one another may be altered Because of their small content of damaged cells it may yet prove that supravital preparations will be the method of choice

Exsanguination of the animal by cutting the abdominal aorta is important not merely to obtain autogenous serum but also in order to drain away from the bone marrow as much blood as possible If the animal is not exsanguinated the amount of residual blood in the vessels may be quite appreciable though some information about it may be obtained by performing both a red cell count and a reticulocyte count on the marrow suspension It is probable that the vast majority of the marrow reticulocytes are red cells which have just been formed in the marrow and are about to be discharged into the blood

Number of Lymphocytes in Bone Marrow

As far as the total number of nucleated cells in the normal marrow is concerned we find they number in the neighbourhood of 1,500 000 per cu mm (1 235 000 Yoffey Metcalf Herdan and Nairn 1951 1 248 000 Hudson Herdan and Yoffey 1952 1 690 000 Yoffey Ancill Holt Owen Smith and Herdan 1954 1 393 000 Harris Ancill Herdan and Yoffey 1954) The absolute figures for the lymphocytes in these four groups of experiments were respectively 145 000 246 000 322 000 and 314 000 per cu mm of marrow

The lymphocytes are virtually all small lymphocytes and the quantitative data make it clear beyond all doubt that they are present in bone marrow in

surprisingly large numbers far too large to be attributable to blood contamination In fact, the lymphocyte population of bone marrow is so high in comparison with blood that the only possible effect of dilution with blood would be to diminish it (Blitstein 1944) Our first quantitative studies were performed on rabbits, in which it could be calculated that the number of lymphocytes present in the marrow was such that it could very easily account for those daily leaving the blood, after entering it via the thoracic duct But a similar quantitative situation prevails as Kindred (1942) has already noted in the wall of the alimentary tract where he calculated that there were three times as many lymphocytes as were present in the circulating blood However in our recent guinea pig experiments based upon a much more accurate technique than was employed in the earlier rabbit series calculations on the lines presented later in this paper indicate that the bone marrow contains something like 10 to 20 times the number of lymphocytes normally present in the circulating blood

The lymphocytes in normal mammalian bone marrow are nearly always diffusely scattered and not arranged in organized nodules except as the result of pathological changes or endocrine imbalance (Kabelitz, 1950) Furthermore, mitoses in marrow lymphocytes are very rare indeed If, therefore, lymphocytes are being formed in the bone marrow, and are not obtaining access to it from the blood the only way in which they can be formed is from a blast cell derived by heteroplastic transformation of a precursor resembling presumably the reticular cell of lymph nodes There is no evidence that this transformation occurs though it is true (*vide infra*) that under certain circumstances, an appreciable number of transitions may be found between small lymphocytes and blast cells In these cases however it is far more probable that the reverse change is occurring namely, the transformation of small lymphocytes into blast cells If the bone marrow is indeed a lymphocytopoietic organ then the number of lymphocytes daily entering the blood would be considerably greater than is indicated by estimations of lymph borne lymphocytes and the problem of the blood lymphocytes would become more perplexing than ever

There seems to be little doubt at the present time about the identity of the small lymphocyte in bone marrow which formerly was confused with the 'primitive cell of Cunningham Sabin and Doan (1925) though at a later date Sabin and Miller (1938) concluded that the morphological criteria for distinguishing between primitive cell and small lymphocyte are not satisfactory With this conclusion Schwind (1950) after a careful study of marrow cells by the supravital technique was also in agreement

If the smal lymphocytes of the bone marrow were in fact primitive cells their presence in the marrow in such large numbers would constitute a singularly difficult problem for since these cells practically never undergo mitosis the marrow must also contain a very large number of primitive cell precursors and this does not appear to be the case

One noteworthy feature about the marrow small lymphocytes (at any rate in our own experimental animals young guinea pigs weighing about 400 gm) is that they really are small recalling more than anything else the small lymphocytes of the thymus (Downey 1948) and the smaller lymphocytes of lymph nodes If the small lymphocytes of the marrow are hematogenous as appears

so highly probable one must assume that it is only the smallest lymphocytes which leave the blood in which the residual lymphocytes will be the larger and possibly older members of the group

The lymphocytes in the blood stream may well be a mixed group of cells Wiseman (1931) attempted to subdivide them into younger and older cells largely on the basis of variations in basophilia Frank and Dougherty (1953) endeavored to separate normal from stress lymphocytes In clinical hematology the tendency as a rule is to regard the small lymphocyte as a mature and senile cell However the small lymphocyte is very radiosensitive (Trowell 1952) and radiosensitivity usually is a property of cells which are actively mitotic (small lymphocytes are not) It is claimed that with the appropriate technique, the small lymphocyte can always be shown to possess a nucleolus (Stockinger and Kellner 1952) The DNA content of small lymphocytes is remarkably high when measured by microspectrophotometric methods and normal lymphocytes possess the diploid chromosome number (Petrakis 1953) Furthermore, the small lymphocyte ha a characteristically active type of movement, which is difficult to reconcile with senility

The Quantitative Study of the Granulocytes

The marrow granulocytes were 393 000 per cu mm in a recent series of guinea pigs (Harris *et al*, 1954 mean of 34 counts) and, of these the majority (329 000) were pseudoeosinophile corresponding to the neutrophile in man Eosinophiles numbered 47 000 per cu mm and basophiles 17 000

Together with these absolute counts per cu mm of marrow, data on marrow volume enable us to estimate tentatively the total granulocyte population of the marrow and to begin to form some idea of the numbers which may be discharged into the blood In this way one may begin to calculate one of the fundamental hemopoietic equations

Marrow volume determinations have been made in the rat by Fairman and Corner (1934) and in the rabbit by Nye (1931–1932) Our own data (Hudson and Yoffey 1954) for the guinea pig of the size used in our work gives results of the same order about two cc of marrow per 100 gm of body weight The guinea pigs employed weigh approximately 400 gm and the marrow even in the long bones i red and actively hemopoietic so that there is approximately eight cc of red marrow in each guinea pig The total marrow granulocytes therefore with a count of 393 000 per cu mm would amount to 3144×10^6 The blood granulocytes in this series were 1 150 per cu mm and blood volume determinations by Doctor A F Rogers of the Physiology Department at Bristol University gave an average figure of 27 cc. in an animal so that the total number of granulocytes in the circulation would be 31×10^6 These figures appear to indicate that in this instance the myeloid reserve in the bone marrow was about 100 times the number of granulocytes present in the blood (Kindred 1942) Furthermore of the marrow granulocytes practically three quarters were mature either with segmented nuclei or with nuclei of band form and almost ready for discharge into the circulation If these mature or nearly mature cells could be mobilized rapidly a leukocytosis of the order of 75 000 per cu mm could presumably develop though how long it lasted would

depend upon a number of factors including the length of time the granulocytes remained in the blood, and the speed with which they could be replaced by the multiplication and maturation of the remaining one fourth of the marrow granulocytes If one assumes that these immature myeloid cells could undergo mitosis twice a day, which seems to be well within the bounds of possibility, a sustained leukocytosis of anything up to 75 000 per cu mm would appear to be within the capacity of normal guinea pig marrow

These rough calculations which we have termed the Myeloid Equation indicate one of the ultimate goals of the quantitative study of the marrow cells and as gaps in our knowledge are filled, it may prove possible to write this equation with a considerably greater degree of accuracy For these first rough calculations all the granulocytes have been counted together With more careful analysis of the available data one may perhaps be able to write separate equations for the different groups of granulocytes Among other major unknown factors may be noted the frequency and the speed of mitosis of the marrow cells, and also the length of time the granulocytes remain in the blood

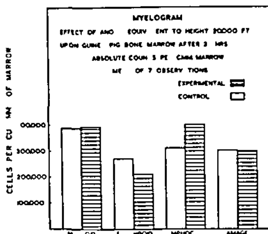
It is clear that in calculations of this kind it is important to have as accurate information as possible about the bone marrow volume We have based our calculations on the data obtained in our own small series of guinea pigs in which the marrow is 2 per cent of the body weight This is a good deal lower than the figures obtainable in man but even so it may well be that 2 per cent is somewhat on the high side However if the calculations are based on a red marrow volume of 1 per cent of the body weight a figure well below all those quoted in the literature the marrow in the present series would still have a very high myeloid reserve, and would contain 10 times as many lymphocytes as are normally present in the blood stream

The Effect of Anoxia on the Bone Marrow

One of the first of our quantitative studies was directed to the effects of anoxia on the bone marrow Our group (Batten *et al* 1953) spent some time last summer at the Jungfraujoch Forschungsstation situated at a height of a little over 11 000 feet to observe the changes which took place in the marrow during the process of adaptation Increased erythropoietic activity occurred as might be expected The other statistically significant change in the marrow was unexpected namely a marked fall in the marrow lymphocytes quite clearly marked during the first five days of adaptation

The full interpretation of this change is not simple But it is worth noting that other observers too have remarked a fall in marrow lymphocytes at the commencement of a period of increased erythropoietic activity such as occurs for example in pernicious anemia when treatment is first begun

It is interesting also to note that exposure to a more violent anoxic stimulus *e g* equivalent to a height of 20 000 feet appears (in some preliminary experiments by Doctor R S Harris) to produce during the first 24 hours an appreciable rise in the marrow lymphocytes as well as a fall in the erythroid cells (FIGURE 1) The full significance of these marrow changes has yet to be evaluated but it is very striking in these activated marrows to observe numerous transitions between small lymphocytes and typical blast cells The change in

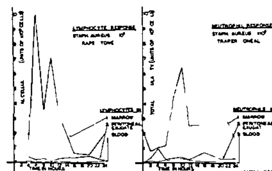


F 1 Th ffect f no th m ll groups in th bo m ow (H rr 1954)

the small lymphocyte is both nuclear and cytoplasmic The nucleus enlarges and acquires the typical leptochromatic appearance of the blast cell nucleus rather than the characteristic pachychromatic configuration of the small lymphocyte nucleus The cytoplasm also gradually increases and becomes more and more basophilic For some time the transforming cell still shows the high nucleus-cytoplasm ratio so evident in the small lymphocyte

The Effect of Bacteria

One of our group Doctor R J Ancill is at present investigating the quantitative changes in the marrow cells following the intraperitoneal injection of vaccines of *H pertussis* and of *Staphylococcus aureus* Though sufficient results are not yet available to meet the requirements of a full statistical analysis the graphs (FIGURE 2) indicate the trend of the preliminary results which Doctor Ancill is obtaining At six hours there is a sharp increase in the number of



Fi 2 Th ff t f gl t pe t l j t f *Staphyl occ au* in on the lymphocyt d t ph l (pse doe ph l) o t t f bo m rrow blood d pe to eal xudat (An ll 1954)

marrow lymphocytes and at ten hours, they decrease in number, but the marrow granulocytes increase

It is of interest that, in these experiments where the vaccine was injected intraperitoneally, a peritoneal exudate formed rich in polymorphs These bodies could be counted and compared with the number of cells discharged from the marrow So far, the sum of the blood polymorphs and of the exudate cells has been well below the number of cells which seem to have been discharged from the marrow If this type of experiment can be extended and confirmed, it should yield valuable information concerning the speed with which leukocytes can leave the marrow for the blood and then escape from the blood in response to chemotactic and other stimuli

Splenectomy and the Bone Marrow

In a small group of experiments which has just been completed (Ensell *et al* 1954), the earlier effects of splenectomy upon the bone marrow have been investigated The histogram (FIGURE 3) is based on the means of seven experiments and a larger series will be required before one can arrive at any final conclusions, but the trend after splenectomy would appear to be quite definitely toward an increase in the total marrow population, with the lymphocytes especially involved in the process It would perhaps have been wiser not to have given these preliminary results at this stage, but I have done so to indicate primarily the way in which our quantitative technique brings out the three major cell groups in the bone marrow

The Blood Span of the Leukocytes

One of the essential data for calculating the factors governing the normal level of the blood leukocytes is the length of time the leukocytes remain in the blood This problem is fundamental and answers to it have been given in a variety of ways with conflicting results

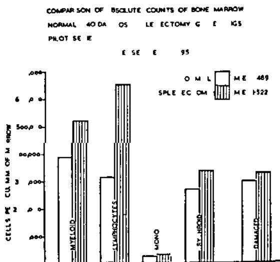


FIGURE 3 The effect of splenectomy on the cell content of bone marrow (Ensell *et al* 1954)

In the case of the lymphocytes calculation of the daily replacement factor suggested a blood span of about 12 hours in the dog (Yoffey 1935 Adams *et al* 1945) and in the rat (Reinhardt 1945) and of five to eight hours in the cat and rabbit (Sanders *et al* 1940)

Another type of experiment consists in actually transfusing cells and observing how quickly they disappear from the blood One of the earliest experiments of this type was that of Minot and Isaacs (1925) who transfused a patient suffering from lymphosarcoma with blood from a patient with chronic lymphatic leukemia The lymphocytes in the recipient s blood increased about threefold but dropped almost to their pretransfusion level within 35 minutes without any evidence of lymphocytes being destroyed while in circulation Erf (1940) injected large numbers of viable heterogenous lymphocytes into rabbits which had been subjected to gastroenterectomy and splenectomy and found that the blood lymphocytes showed either a marked fall from the outset or a slight initial rise which had disappeared in a few hours Lawrence Ervin and Wetrich (1945) rendered cats leukopenic by irradiation and then transfused leukocytes from a normal cat by cross circulation They concluded that the white cells remained in the blood 16 to 24 hours

White (1954), transfusing blood whose white cells had been tagged with atabrine dihydrochloride found that as a rule these cells disappeared from the recipient s blood in 30 to 90 minutes though in leukemic patients they persisted longer Van Dyke and Huff (1951) employed parabiotic rats one of which was rendered anemic by exposure to X rays They calculated that leukocytes remained in the blood for about three hours Farr (1951) in rabbits gave autogenous lymphocytes which had been labeled with a nontoxic acridine dye and found that most of the lymphocytes left the blood in two hours for the bone marrow or lymphatic tissues In the bone marrow the lymphocytes appeared to be transforming into myelocytes Osogoe (1950) used suspensions of lymphocytes made from the mesenteric lymph glands of 3 to 5 rabbits and injected them into another rabbit He found that they disappeared from the blood within 2 to 3 hours, but it is doubtful as he himself points out whether his experiments can be regarded as throwing light on the normal fate of the lymphocyte Weisberger *et al* (1951) found that large numbers of rat lymphocytes injected into other rats disappeared from the blood in a few minutes As in all these experiments the question at once arises whether leucocytes which have been taken out of their normal *milieu* and subjected to even slight manipulation can be regarded as normal Weisberger *et al* obtained lymphocytes from rats which had been injected with P_{32} 24 hours previously and after injecting these radioactive lymphocytes found that 28 per cent of the radioactivity appeared in the lungs 23 per cent in the liver 4 per cent in the spleen and 5 per cent in the kidney

The transfusion experiments of Fichtelius (1953) led him to think more especially of the spleen as a lymphocyte depot though here again he was dealing with lymphocytes obtained either from a completely different species or in the case of syngenesio-transfusion (litter mates) possibly modified by the process of preparing the suspension

White (1954) transfused blood containing leukocytes tagged with atabrine and concluded that the transfused leukocytes disappeared from the blood of the recipient in from 30 to 90 minutes White emphasized the concept of second ary leukocyte equilibria The primary equilibrium would be that concerned with the formation of leukocytes their passage into the blood, and their dis appearance from the blood after a given lapse of time The secondary equilib rium would be that in which leucocytes which had been discharged into the blood from the hemopoietic organs would repeatedly pass out of the blood and return to it As White says ' one may further theorize that leukocytes may have many intravascular life spans which may be only a fraction of the life span of the cell

A different approach to the problem is that adopted by Ottesen (1948) and later workers Here the animal is given P_{32} which is then incorporated in presumably stable form in the DNA of the newly forming blood cells These cells can then be detected when the mature cells are discharged into the blood If the P_{32} does not impair the development or activity of the cells in any way this type of cell marking ought to give very satisfactory results Ottesen (1948) found in the hen, the peak of activity in the blood leucocytes at 4 to 5 days, gradually tapering off to the 15th day Kline and Clifton (1952–1953) working with man estimated the total life span of the leukocytes as a whole to be about 13 days of which the first four days were spent in the bone marrow This type of figure is very much higher than the blood span as calculated by the older methods The position becomes even more complex as a result of the work of Osgood Tivey *et al* (1952) from which it appears that the blood span may vary appreciably from three days in cases of chronic granulocytic or subacute monocytic leukaemia to 30 days in chronic lymphatic leukemia It may well be that even in healthy individuals the blood span of the leukocytes is subject to a good deal of variation and for the time being it may not be possible to work out the full Myeloid Equation To begin with therefore our group is concentrating its efforts in the main on the production and discharge of cells from the bone marrow

Summary

Problems involved in the quantitative study of leukocyte production are dis cussed in relation to both the lymphocytes and the granulocytes Because of their association with the lymphatic vessels the lymphocytes have been esti mated quantitatively before it has been possible to obtain comparable data for the granulocytes

A technique is now described for the quantitative study of the nucleated cells of the bone marrow in guinea pigs and the data thus obtained are considered in relation to the problem of the destination of the blood lymphocytes and in con nection with the discharge of granulocytes into the blood In the normal animal quantitative estimations emphasize the great myeloid reserves of the marrow

A preliminary report is given of the response of the bone marrow to anoxia splenectomy and bacteria

Bibliography

1 ADAMS W S R H SAUNDERS & J S LAWRENCE 1954 Output of lymphocytes in cats including studies on thoracic duct lymph and peripheral blood Am J Physiol 144 29–304
2 ALLEN L 1945 A quantitative study of tissue fluid—lymph cellular ratios Anat Record 92 2 9–288
3 ANCILL, R J 1954 Unpublished data
4 BATTEN E H W J GALL, G HALLEY R S HARRIS A F ROGERS & J M YOFFEY 1953 Unpublished data
5 BLALOCK, A C S ROBINSON R S CUNNINGHAM & M F GRAY 1937 Experimental studies on lymphatic blockage Arch Surg 34 1049
6 BLITSTEIN J 1944 Considerations sur l origine des lymphocytes de la moelle osseuse de l'homme Compt rend soc biol Paris 139 331–334
7 CRAFTS R C 1941 The effects of endocrines on the formed elements of the blood II The effect of estrogens in the dog and monkey Endocrinology 29 606
8 CRAFTS, R C 1948 The effects of estrogens on the bone marrow of adult female dogs Blood 3 2 6–285
9 CUNNINGHAM R S F R SABIN & C A DOAN 1925 The development of leucocytes lymphocytes and monocytes from a specific stem-cell in adult tissues Carnegie Inst Wash Pub Contrib Embryol 16 227
10 DOUGHERTY T F & L F KUMAGAI 1951 Influence of stress stimuli on lymphatic tissue of adrenalectomized mice Endocrinology 48 691–699
11 DOUGHERTY T F & A WHITE 1944 Influence of hormones on lymphoid tissue structure and function The role of the pituitary adrenotrophic hormone in the regulation of the lymphocytes and other cellular elements of the blood Endocrinol. 35 1–14
12 DOUGHERTY T F & A WHITE 1945 Functional alterations in lymphoid tissue induced by adrenal cortical secretion Am J Anat 77 81–116
13 DOUGHERTY T F & A WHITE 1947 An evaluation of alterations produced in lymphoid tissue by pituitary adrenal cortical secretion J Lab Clin Med 32 584–605
14 DOWNEY H 1948 Cytology of rabbit thymus and regeneration of its thymocytes after irradiation Blood 3 1315–1341
15 DRINKER C K & J M YOFFEY 1941 Lymphatics Lymph and Lymphoid Tissue Harvard Univ Press Cambridge Mass
16 EHRICH W F D L DRABKIN & C FORMAN 1949 Nucleic acids and the production of antibody by plasma cells J Exptl Med 90 157 168
17 ENSELL F J R S HARRIS & J M YOFFEY 1954 Unpublished data
18 ERF L A 1940 The disappearance of intravenously injected lymphocytes in the absence of the gastro-intestinal tract Am J Med Sci 200 1–11
19 FAIRMAN E & G W CORNER 1934 The bone marrow volume of the albino rat Anat Record 60 1–4
20 FARR R.S 1951 Experiments on the fate of the lymphocyte Anat Rec 109 515–534
21 FEIN W M & A S GORDON 1950 Influence of hypophysectomy on hemopoietic response of rats to lowered barometric pressures Endocrinology 47 364–369
22 FICHTELIUS K E 1953 On the fate of the lymphocyte Acta Anat (Suppl 19 1 ad) 19 1–78
23 FRANK J A & T F DOUGHERTY 1953 The assessment of stress in human subjects by means of quantitative and qualitative changes of blood lymphocytes. J Lab Clin Med 42 538 549
24 GLENN, W L F N BAUER & S L CRESSON 1949 Experimental thoracic duct fistula Blood 4 1338–1345
25 GORDON A S 1939 Studies in bone marrow J Lab Clin Med 24 357
26 GORDON A S & H A CHARIPPER 194 The endocrine system and hemopoiesis Ann N Y Acad Sci 48 615–641
26a GORDON A S & G F KATSH 1949 The relation of the adrenal cortex to the structure and phagocytic activity of the macrophagic system Ann N Y Acad Sci 52 1 30
27 GORDON A S S J PILIERO & D LANDAU 1949 The relation of the adrenal to blood formation in the rat Endocrinology 49 497–511
28 HARRIS R S, G HERDAN R J ANCILL & J M YOFFEY 1954 A quantitative comparison of the nucleated cells in the right and left humeral bone marrow of the guinea pig Blood 9 374–378
29 HUDSON G G HERDAN & J M YOFFEY 1952 Effect of repeated injections of ACTH upon the bone marrow Brit Med J 1 999–1001
30 HUDSON G & J M YOFFEY 1954 Unpublished observations
31 KABELITZ H J 1950 Klinische und hämatologische Beobachtungen zur Entwicklung lymphatischer Keimzentren im Knochenmark Acta haematol. 3 347–366

32 KINDRED J E 1940 A quantitative study of the hemopoietic organs of young albino rats Am J Anat 67 99–149
33 KINDRED J E 1942 A quantitative study of the hemopoietic organs of young adult albino rats Am J Anat 71 207–243
34 KLINE D L & E E CLIFFTON 1952 Life span of leukocytes in man J Applied Physiol 5 79–84
35 LAWRENCE J S D M ERVIN & R M WETRICH 1945 Life cycle of white blood cells Am J Physiol 144 284–296
36 MANN J D & G M HIGGINS 1950 Lymphocytes in thoracic duct intestinal and hepatic lymph Blood 5 177–190
37 MINOT G R & R ISAACS 1925 Transfusion of lymphocytes J Am Med Assoc 84 1713–1715
38 NYE R N 1931–32 Bone marrow volume in rabbits Proc Soc Exptl Biol Med 29 34–38
39 OSGOOD E E H TIVEY K B DAVISON A J SEAMAN & J G LI 1952 The relative rates of formation of new leucocytes in patients with acute and chronic leukemias Cancer 5 331–335
40 OSOGOE B 1950 Transplantation of hematopoietic tissues into the circulating blood I Experiments with lymph nodes in normal rabbits Anat Record 107 193–219
41 OTTESEN J 1948 Life span of red and white blood corpuscles of the hen Nature 162 730–731
42 PETRAKIS N L 1953 Microspectrophotometric estimation of the desoxyribonucleic acid (DNA) content of individual normal and leukemic human lymphocytes Blood 8 905–915
43 REINHARDT W O 1946 Growth of lymph nodes thymus and spleen and output of thoracic and cervical duct lymphocytes in the normal rat Anat Record 94 197–211
44 SABIN F R & F R MILLER 1938 Normal bone marrow In Handbook of Hematology 3 1789 H Downey Ed Hoeber New York N Y
45 SANDERS A G H W FLOREY & J M BARNES 1940 The output of lymphocytes from the thoracic duct in cats and rabbits Brit J Exptl Path 21 254–263
46 SANTISTEBAN G A & T F DOUGHERTY 1954 Comparison of the influences of adrenocortical hormones on the growth and involution of lymphatic organs Endocrinology 54 130–146
47 SCHULTZE W 1925 Untersuchungen über die Capillaren und postcapillären Venen lymphatischer Organe Z Anat Entwicklungsgeschichte 76 421
48 SCHWIND J L 1950 The supravital method in the study of blood and bone marrow cells Blood 5 597–622
49 SJOVALL H 1936 Experimentelle untersuchungen über das Blut und die blutbilden den Organe—besonders das lymphatische Gewebe—des Kaninchens bei wiederholten Aderlassen Hakan Ohlssons Bokryckeri Lund Sweden
50 STOCKINGER L & G KELLNER 1952 Der Lymphozyten nukleolus I Mitteilung Die Darstellung und Bedeutung des Nukleolus Wien Z inn Med 33 135–141
51 TROWELL O A 1952 The sensitivity of lymphocytes to ionizing radiation J Path Bact 64 687–704
52 VAN DYKE D C & R L HUFF 1951 Life span of white blood cells as measured in irradiated parabiotic rats Am J Physiol 165 341–347
53 WEISBERGER A S R W HEINLE J P STORAASLI & R HANNAH 1951 Transfusion of leukocytes labelled with radioactive phosphorus J Clin Invest 29 336–341
54 WEISKOTTEN H G 1930 The normal life span of the neutrophile (amphophile) leukocyte (rabbit) Am J Path 6 183–190
55 WHITE L P 1954 The intravascular life span of transfused leukocytes tagged with atabrine Blood 9 73–82
56 WISEMAN B K 1931 Criteria of the age of lymphocytes in the peripheral blood J Exptl Med 54 271
57 YOFFEY J M 1935 Variation in lymphocyte production J Anat 70 507
58 YOFFEY J M 1950 The mammalian lymphocyte Biol Revs Cambridge Phil Soc 25 314–343
59 YOFFEY J M R J ANCILL J A G HOLT B OWEN SMITH & G HERDAN 1954 A quantitative study of the effects of Compound F Compound E & Compound A upon the bone marrow of the guinea pig J Anat 88 115–130
60 YOFFEY J M & C K DRINKER 1939 The cell content of peripheral lymph and its bearing on the problem of the circulation of the lymphocyte Anat Record 74 417
61 YOFFEY J M W K METCALF G HERDAN & V NAIRN 1951 Effect of ACTH and suprarenal extract on the bone marrow Brit Med J 1 660–667
62 YOFFEY J M & J PARNELL 1944 The lymphocyte content of rabbit bone marrow J Anat 78 109–112

Part IV Defense Functions of the Leukocytes

CHEMOTAXIS AND LOCOMOTION OF LEUKOCYTES

By Morton McCutcheon

Department of Pathology University of Pennsylvania School of Medicine Philadelphia Pa

By chemotaxis[6] is meant a directional response of the organism in this case the leukocyte to chemical substances in its environment Chemotaxis is said to be positive if the cell moves toward higher concentrations of such substances negative if it moves away from higher concentrations Chemotaxis is absent or indifferent if there is no directional response to the presence of the substance

It should be emphasized that chemotaxis is manifested by the *direction* of movement not by its velocity When there is no known chemotactic substance in the field the leukocyte may move as rapidly as when it is responding to a chemotactic stimulus

A convenient method[7] for studying chemotaxis is to obtain a suspension of granulocytes by injecting salt solution into the periotoneal cavity of an animal such as the rabbit When the fluid is withdrawn after a few hours it is centrifuged and the precipitated cells are suspended in plasma To elicit chemotaxis a minute clump of bacteria or other particles is placed on a glass slide and allowed to dry A drop of the cell plasma suspension is superimposed and allowed to spread between slide and coverslip This preparation after being sealed with paraffin is viewed on the warm stage of a microscope The microscopic image of the cell may be projected by a device such as a camera lucida and the position of the cells recorded at intervals or movements of cells may be recorded by motion pictures

Granulocytes begin to move at once If no chemotactic substance is present their motion is at random the cells frequently changing direction but if a clump of bacteria or collodion particles is in the field the cells immediately move toward these particles If on the contrary certain other particles such as kaolin or aluminum silicate are present all the cells move away Such a negative response may be just as strong as the positive reaction to bacteria[7]

The kinds of leukocytes which show chemotaxis best are the granulocytes that is neutrophilic and eosinophilic[4] polymorphonuclears No information is available about the reactions of basophils

Lymphocytes on the contrary do not show chemotaxis at least under these conditions[3] They move about half as fast as granulocytes but their direction is not affected by bacteria or other material as far as known They may be studied conveniently by placing a drop of blood on a glass slide and causing it to spread between slide and coverslip Under these conditions lymphocytes generally do not move at once but only after minutes or even hours differing in this respect from polymorphonuclears In tissue cultures such as of lymph nodes however locomotion is not thus delayed

In monocytes and macrophages locomotion is delayed even more In blood or exudate *in vitro* these cells are stationary beginning to move only after three or four days incubation[8] However in cultures of tissue such as omen

tum locomotion begins promptly[1] and when fragments of buffy coat are planted in plasma, monocytes move out from the fragment though somewhat later than do the granulocytes Probably differences in delay of locomotion depend on cellular aggregation if macrophages are dispersed, locomotion is delayed whereas if they are aggregated locomotion begins soon An easy way to test the validity of this generalization would be to centrifugalize exudative macrophages, and then to compare their locomotion with that of dispersed cells

These observations suggest that macrophages require accumulated metabolic products before motion is possible, and the same thing may be true of lymphocytes Granulocytes evidently need no such stimulus or else the stimulating substance is always at hand

Chemotaxis of monocytes varies with *in vitro* conditions In explants of omentum emigrating macrophages are not attracted by bacteria or collodion particles both of which strongly attract granulocytes, but macrophages are repelled by silicate particles but not strongly [1] According to a recent report,[3] blood monocytes after a week s incubation were strongly attracted by bacteria Chemotaxis may be manifested in another way that is, by thrusting out pseudopods to engulf nearby cells as if the macrophage had been chemically attracted

Of the various substances which stimulate chemotactic responses in granulocytes products of injury have been investigated especially by Menkin [8] This investigator has shown the importance of polypeptides (leukotaxin) and it may be that *in vivo* substances of this character are of paramount importance in chemotaxis * Some other investigators[5] regard polysaccharides such as glycogen as the important natural chemotactic agent Monosaccharides are reported to attract granulocytes from starved rats but not from fed rats

In *in vitro* experiments I have found particles (bacteria collodion silicates and others) useful in semiquantitative experiments designed to investigate the nature of the chemotactic field I shall now describe some of these experiments the purpose of which was to find out in what sort of field leukocytes show directional response to particles At this time information on this subject is far from complete

When we examine a camera lucida record of granulocytes reacting to bacteria or collodion particles cells at the periphery of the field are seen to move less directly in more tortuous sometimes looping paths than do cells closer to the source of attraction When microscopic fields farther away from the target are observed leukocytes are found to follow still more devious paths At a distance of about 1 mm as many cells move away from the target at any one time as toward it indicating absence of chemotaxis From these observations there is clearly a gradient of stimulus most intense at the target decreasing with distance becoming imperceptible at about 1 mm Characterizing this gradient the first possibility to consider is a concentration gradient of a substance that diffuses from the particles *e g* of bacteria But does the chemo

Fo d ff rent p see Harr s

tactic agent diffuse? Opposed to this explanation are the following observations

The chemotactic response is of maximal intensity as soon as the preparation is made or as soon as the cells are brought to a suitable temperature There after the directness of cell paths does not tend to increase or decrease as long as leukocytes move well Also the size (diameter) of the chemotactic field does not change with time as measured by the percentage of cells moving toward the target from a given distance From these observations a diffusible attracting or repelling substance is regarded as unlikely

One may now ask Is any chemotactic substance at all given off by the particles? If so the chemotactic effect of particles should be decreased by repeated washings This expectation however, is disproved by washing bacteria or particles of collodion or of aluminum silicate After any number of washings these particles attract (or repel) leukocytes as strongly as before Therefore either there is no soluble chemotactic substance present or its supply cannot be exhausted

A second reason for doubting that such particles give off chemotactic substances is that these particles especially collodion and Lloyd s reagent (aluminum silicate) are almost insoluble in blood Consequently at 100 microns from a clump of particles a distance at which chemotaxis is often active the number of dissolved molecules must be exceedingly small too small it would seem to set up an effective concentration gradient

Finally it has not been possible to extract from Lloyd s reagent any substance that would produce chemotaxis

The evidence accordingly opposes the idea that particles such as those mentioned give off chemotactic substances

Instead of a field depending on chemical gradients the field in which leukocytes react may be conceived to result from electric charges on the particles This supposition is unlikely if one takes into account the relatively long distances (several hundred microns) over which the particles act Notwithstanding experiments of several types were devised that might distinguish between electrical and chemical fields but these experiments did not support the hypothesis of an electrical field

A different explanation is possible instead of giving off chemotactic substances particles may differentially adsorb substances from the surrounding plasma In this way the particles would set up a concentration gradient consisting of plasma substances Consistent with this hypothesis is the strong adsorptive ability of collodion particles and of Lloyd s reagent Preliminary experiments suggest that this hypothesis is worth testing

Summary

Chemotaxis the directional response of organisms or cells to substances in their environment is termed positive or negative depending on whether there is attraction or repulsion Of leukocytes chemotaxis is displayed especially by granulocytes whereas monocytes and macrophages react only weakly to chemotactic stimuli under most conditions As far as known chemotaxis is not shown

tum, locomotion begins promptly[1] and when fragments of buffy coat are planted in plasma monocytes move out from the fragment though somewhat later than do the granulocytes Probably differences in delay of locomotion depend on cellular aggregation if macrophages are dispersed locomotion is delayed, whereas if they are aggregated locomotion begins soon An easy way to test the validity of this generalization would be to centrifugalize exudative macrophages, and then to compare their locomotion with that of dispersed cells

These observations suggest that macrophages require accumulated metabolic products before motion is possible and the same thing may be true of lymphocytes Granulocytes evidently need no such stimulus or else the stimulating substance is always at hand

Chemotaxis of monocytes varies with *in vitro* conditions In explants of omentum emigrating macrophages are not attracted by bacteria or collodion particles both of which strongly attract granulocytes but macrophages are repelled by silicate particles but not strongly [1] According to a recent report[2] blood monocytes, after a week's incubation were strongly attracted by bacteria Chemotaxis may be manifested in another way that is by thrusting out pseudopods to engulf nearby cells as if the macrophage had been chemically attracted

Of the various substances which stimulate chemotactic responses in granulocytes products of injury have been investigated especially by Menkin[3] This investigator has shown the importance of polypeptides (leukotaxin') and it may be that *in vivo* substances of this character are of paramount importance in chemotaxis * Some other investigators[5] regard polysaccharides such as glycogen as the important natural chemotactic agent Monosaccharides are reported to attract granulocytes from starved rats but not from fed rats

In *in vitro* experiments I have found particles (bacteria collodion silicates and others) useful in semiquantitative experiments designed to investigate the nature of the chemotactic field I shall now describe some of these experiments the purpose of which was to find out in what sort of field leukocytes show directional response to particles At this time information on this subject is far from complete

When we examine a camera lucida record of granulocytes reacting to bacteria or collodion particles cells at the periphery of the field are seen to move less directly in more tortuous sometimes looping paths than do cells closer to the source of attraction When microscopic fields farther away from the target are observed leukocytes are found to follow still more devious paths At a distance of about 1 mm as many cells move away from the target at any one time as toward it indicating absence of chemotaxis From these observations there is clearly a gradient of stimulus most intense at the target decreasing with distance becoming imperceptible at about 1 mm Characterizing this gradient the first possibility to consider is a concentration gradient of a substance that diffuses from the particles *e g* of bacteria But does the chemo-

Fo diffe t op se H rr s

PHAGOCYTOSIS

By Claude Starr Wright and Matthew C Dodd

Departments of Medicine and Bacteriology Ohio State University Columbus Ohio

In 1870 Langhans observed that leukocytes phagocyted fragments of worn out red blood cells found in tissue debris Panum (1874) and Rosser (1881) suggested that this process might actually be a method of destruction of microbes[1] It remained for Metchnikoff to establish phagocytosis firmly as an important defense mechanism In 1882 Metchnikoff then a Russian political refugee living in Messina Sicily, began his studies His first experiment was made by placing a rose thorn under the skin of the starfish larva This organism having no blood vessels or nervous system responded by surrounding the thorn with mobile cells[2] Metchnikoff proposed that cellular phagocytosis was the primary means of the body defense mechanism This suggestion was not enthusiastically received by the humoral school of investigators For the past 70-odd years the relative values of the *humoral* versus the *cellular* defense phases have been argued The humoral aspects have received more attention on the whole and have been assigned a greater relative importance Undoubtedly one of the major factors was the facile means of demonstrating antibodies experimentally It has been encouraging to see the renewed interest that has been stimulated in the last few years in the cellular phases

The phagocytic defense mechanism by leukocytes consists of the three phases chemotaxis phagocytosis *per se* and digestion of the ingested material This discussion will be concerned with the second phase that is phagocytosis *per se* Our purpose is twofold first to review the elements involved including the pertinent factors influencing these elements and second to comment on the theoretical mechanisms underlying this process The limitations imposed are obvious Mudd McCutcheon and Lucké made a masterful review of phagocytosis in 1934[3] Berry and Spies brought the subject up to date with their excellent survey in 1949[4] For the most part we shall confine our discussion to observations that have been reported in the intervening years

The specified functions of the macrophage and microphage systems are not as distinctly categorized as Metchnikoff first reported The functions of these two systems overlap to a great degree and are so closely coordinated that one system may easily compensate for the other The fundamental mechanisms involved in both cell systems are similar their differences being primarily geographic rather than physiologic Information gained experimentally from a study of the one system has frequently been applied to the other with success It is therefore difficult to separate validly the two systems either by function or by mode of action

The three elements involved in the phagocytic phenomena are (1) the phagocyte (2) the ingested particle and (3) the environment in which ingestion takes place Discussion is presented in this order

The Phagocyte

Of the mature leukocytes the granulocytes and monocytes are potentially phagocytic The neutrophilic granulocytes are more phagocytic than the

by lymphocytes Among various substances that attract or repel leukocytes the best known are products of tissue injury and particles such as bacteria, collodion and silicates Certain aluminum silicates are powerful repelling agents for leukocytes *in vitro*, whereas bacteria and collodion attract them Still other particles, such as carbon, have no chemotactic effect When a minute clump of chemotactically active particles is placed on a glass slide, and a suspension of leukocytes in blood or plasma is superimposed and spread beneath a coverslip, a chemotactic field is set up of microscopic size The properties of this field have been explored in an attempt to learn something of the mechanism of chemotaxis A gradient of some kind exists, but that it is a gradient of substances diffusing from the test particles is doubted Since repeated washing of particles does not decrease their chemotactic effect, and as the particles are almost insoluble in plasma, it seems doubtful that any chemotactic substance is given off by the particles Also, it has not proved possible to extract a chemotactic substance from the particles Alternative explanations of the mechanism are that the chemotactic field is of an electrical nature or is set up by differential adsorption of plasma constituents to the test particles These possibilities are being explored

References

1 COMAN D R 1940 Arch Path 30 896
2 DIXON H M & M MCCUTCHEON 1935 Arch Path 19 679
3 HARRIS H 1953 Brit J Exptl Path 34 276
4 INGRAHAM E S & W B WARTMAN 1939 Arch Path 28 318
5 KUNA A & R CHAMBERS 1953 J Clin Invest 32 436
6 MCCUTCHEON M 1946 Physiol Revs 26 319
7 MCCUTCHEON M D R COMAN & H M DIXON 1939 Arch Path 27 61
8 MENKIN V 1938 J Exptl Med 67 129 145 153
9 HARRIS H 1954 Physiol Revs 54 529

PHAGOCYTOSIS

By Claude Starr Wright and Matthew C Dodd
Departments of Medicine and Bacteriology Ohio State University Columbus Ohio

In 1870 Langhans observed that leukocytes phagocyted fragments of worn out red blood cells found in tissue debris Panum (1874) and Rosser (1881) suggested that this process might actually be a method of destruction of microbes[1] It remained for Metchnikoff to establish phagocytosis firmly as an important defense mechanism In 1882 Metchnikoff then a Russian political refugee living in Messina Sicily began his studies His first experiment was made by placing a rose thorn under the skin of the starfish larva This organism having no blood vessels or nervous system responded by surrounding the thorn with mobile cells[2] Metchnikoff proposed that cellular phagocytosis was the primary means of the body defense mechanism. This suggestion was not enthusiastically received by the humoral school of investigators For the past 70-odd years the relative values of the *humoral* versus the *cellular* defense phases have been argued The humoral aspects have received more attention on the whole and have been assigned a greater relative importance Undoubtedly one of the major factors was the facile means of demonstrating antibodies experimentally It has been encouraging to see the renewed interest that has been stimulated in the last few years in the cellular phases

The phagocytic defense mechanism by leukocytes consists of the three phases chemotaxis phagocytosis *per se* and digestion of the ingested material This discussion will be concerned with the second phase that is phagocytosis *per se* Our purpose is twofold first, to review the elements involved including the pertinent factors influencing these elements and second to comment on the theoretical mechanisms underlying this process The limitations imposed are obvious Mudd McCutcheon and Lucke made a masterful review of phagocytosis in 1934[3] Berry and Spies brought the subject up to date with their excellent survey in 1949[4] For the most part we shall confine our discussion to observations that have been reported in the intervening years

The specified functions of the macrophage and microphage systems are not as distinctly categorized as Metchnikoff first reported The functions of these two systems overlap to a great degree and are so closely coordinated that one system may easily compensate for the other The fundamental mechanisms involved in both cell systems are similar their differences being primarily geographic rather than physiologic Information gained experimentally from a study of the one system has frequently been applied to the other with success It is therefore difficult to separate validly the two systems either by function or by mode of action.

The three elements involved in the phagocytic phenomena are (1) the phagocyte (2) the ingested particle and (3) the environment in which ingestion takes place Discussion is presented in this order

The Phagocyte

Of the mature leukocytes the granulocytes and monocytes are potentially phagocytic The neutrophilic granulocytes are more phagocytic than the

eosinophils and basophils Most observers agree that the mature lymphocyte is not phagocytic in the same sense However there is some difference of opinion [5, 6, 7] There is also conflicting opinion regarding the phagocytic potentiality of plasma cells [8] Marked erythrophagocytosis by plasma cells from a case of plasma cell leukemia was recently reported [9]

The younger mature granulocytes with one or two nuclear lobes are the most effective phagocytes [10] Granulocytes from anemic humans[11] and anemic experimental animals[1] show *increased* phagocytic potential Similar enhancement is seen after granulocytes have been treated with surface active agents [13] Diminished phagocytic activity has been shown by granulocytes from (1) infants [14] (2) from myeloid leukemia patients [15] (3) from animals made deficient of any of the B vitamins except inositol and p-aminobenzoic acid (Mills) [16] of vitamin C (Mills Nungester and Ames) [16, 17] or of protein (Mills),[16] (4) from patients on corticotropin or cortisone therapy,[18] and (5) from stored blood Leukocytes suspended in albumin solution assume a spherical form and remain discrete with no tendency to clump or undertake phagocytosis [19]

The phagocytic capacity of leukocytes is dormant below 15 C Above this temperature their activity increases with the approach to 37 C Above 40 to 42° C it again diminishes [20, 21]

The Particle

Observations on phagocytosis are most frequently made by noting the ingestion of cells such as bacteria red blood cells or other debilitated body cells and of noncellular foreign particulate matter including crystals and nonmiscible vacuolated liquids

The production of antiphagocytic factors by bacteria actually may inhibit their ingestion and in fact this capacity is frequently associated with bacterial virulence or the ability to parasitize and invade animal tissues This is exemplified by certain pneumococcus capsular polysaccharides which are nontoxic and provide the cells with protective surface [22] In contrast the antiphagocytic action of the somatic O antigens on the surface of many gram negative species and of the exotoxins and leukocidins of many gram positive agents is associated with their toxicity for phagocytic cells Nonbacterial substances such as mucin [3] and certain surface active agents[13] may also be antiphagocytic

The capacity of immune sera to enhance phagocytosis of virulent organisms has been known practically since the time of Metchnikoff as the opsonic function of antibody The opsonic action of fresh normal sera is equally well recognized and is usually considered a function of normal antibody plus complement or because of its heat lability due to complement alone Nonspecific opsonic agents include nonantibody proteins such as globin[23] and fibrin Knisely has emphasized the necessity of a fibrinlike coating of blood proteins on inert particles such as India ink and kaolin for their phagocytosis [24] Substances such as tannin enzymes such as trypsin certain viruses and periodate also enhance the phagocytosis of erythrocytes in the presence of normal serum but not in saline [25] It has been shown in this laboratory that increased phagocytosis of enzyme or virus-treated red blood cells is mediated by normal antibody

in normal sera for antigens made available on the surface of the cells by these agents which by themselves, are not the actual opsonins [26, 27, 28]

The Environment

The *environment* in which phagocytosis takes place has received the least attention of the three phases until the last few years Most of the standardized tests for phagocytosis have been made in glass tubes or on smooth surfaces of glass slides or cover slips Physically these *in vitro* systems have little in common with *in vivo* processes The recent discriminating observations by Wood and associates have brought the environmental aspects of phagocytosis into their rightful prominence and perspective [2, 29, 30, 31]

First, Wood makes an important distinction between acute and chronic infections noting that most bacteria causing acute infections in man do damage to the body primarily when they are outside of the cell Once ingested by phagocytes they are destroyed by the enzymes in the phagocytic cells On the other hand most bacteria which cause chronic infections are essentially intracellular organisms

In the normal animal microphagocytes (granulocytes) move intravascularly with the current of blood along the vessel wall in rolling fashion and are not actively phagocytic The entrance of bacteria into the blood stream however causes an immediate change The granulocytes stick to the endothelium of capillaries and assume ameboid motility They are then potentially phagocytic Intravascular phagocytosis without specific opsonization may be accomplished in three ways (1) the granulocyte traps the particle against the endothelium (2) intercellular surface phagocytosis may be caused by a particle trapped between two adjacent leukocytes and (3) small fibrin deposits on the capillary walls catch bacteria and the granulocytes then phagocytize the particles caught in the interstices Intravascular phagocytosis varies inversely with the rate of blood flow It is rare where the flow is rapid and is most frequent in capillaries with sluggish flow

Surface phagocytosis depends on the presence of a relatively rough or sticky surface against which the leukocyte can pin the bacteria It operates best in relatively compact tissues as spleen lymph nodes liver subcutaneous tissue and lungs It is less effective in open cavities where chance contact is less

Most of the common bacteria causing acute infections in man are susceptible to the surface phagocytosis mechanism The principal exception is the pneumococcus type III [23]

Thus the observations of phagocytosis in an environment which simulates closely that of an *in vivo* situation has been emphasized

There are in addition many chemical and physical influences which have already been excellently reviewed [3, 4] We wish to emphasize here only two of these factors (1) corticotropin which lessens phagocytosis [32] and (2) the folic acid antagonists in bone marrow cultures which seemingly enhance phagocytosis [33]

Mechanism

What determines whether a particle is to be phagocytized or not? There is no completely satisfactory answer to this question The theoretical mechanisms have been classically ascribed to phenomena associated with surface energy and electrostatic charge[3, 4, 34, 35]

In attempting to substantiate Fenn's theoretical hypothesis, Berry and his associates found that five of 52 surface active agents tested *in vitro* enhanced phagocytosis when the phagocyte was treated but lessened phagocytosis when the particle was treated[15] Intraperitoneal injection of one of these surface active agents (Triton N — 100) failed to protect mice against experimentally induced infections with *Salmonella* The explanation of this finding was that the effects on the phagocyte and on the particle negated each other Nungester and his associates also found that cationic quaternary ammonium compounds stimulated phagocytosis[36] They found however, that closely related chemical substances which were not surface active behaved similarly They concluded that there was little relation between the lowering of surface tension and the ability to stimulate phagocytosis

It should be added here that no satisfactory methods of determining interfacial tension between surfaces of the phagocyte and the particle are known at present

In 1921 Northrop and De Kruif noted that specific immune serum decreased the electrostatic charge on bacteria[37] We have found that various modifications of erythrocytes by trypsin or virus treatment also lower the electrophoretic mobility of these cells These cellular particles" with lessened charge show a greater susceptibility to phagocytosis There are other factors, however, which also may enhance phagocytosis without measurable alteration of the charge[38]

Nungester and his associates noted that their test leukocytes were electronegatively charged[39] This observation has resulted in their questioning charge as a significant part of the phagocytic mechanism in that electronegatively charged bacteria are attracted to and phagocytized by electronegatively charged leukocytes This phenomenon is a puzzle If means were available for measuring a localization of charge on the phagocyte surface rather than having to measure the total surface charge as i now done these theoretical mechanisms might be more tenable There is evidence that the charges on a cell surface may vary in a mosaic pattern

It has been suggested that the fundamental process of phagocytosis may not be primarily physical but rather biochemical[3, 36, 39] This suggestion makes a challenging area for future exploration

References

1 Mettler C C 1947 History of Medicine 265 Blakiston New York N Y
Wood W B Jr 1951 White Blood Cells v Bacteria Sci American 184 48
3 Mudd S M McCutcheon & B Lucké 1934 Phagocytosis Physiol Revs 14 210
4 Berry L J & T D Spies 1949 Phagocytosis Medicine 28 239
5 Bergel S 1921 Die Lymphocytose ihre experimentelle Begrundung und biologisch klinische Bedetung Springer Berlin Germany

6 Doskocil, A 1923 Phagocytosis in Tuberculosis Časopis lékaru českých Sokolská tr č. 31 Praha 2
7 Rebuck J W R W Smith & R R Margulis 1951 The modification of leukocytic function in human windows by ACTH Gastroenterology 19 644
8 DuBois A H 1938 A propos de la cytologie du myelome multiple Helv Med Acta 5 550
9 Butterworth C E Jr W B Fromeyer Jr & W H Riser Jr 1953 Erythrophagocytosis in a case of plasma cell leukemia Blood 8 519
10 Saslaw, S & C A Doan 1947 The role of phagocytosis in resistance as related to age of granulocytes following primary and secondary reinfection studies with hemolytic streptococci in Macacus Rhesus J Lab Clin Med 32 878
11 Berry L J R M Leyendecker & T D Spies 1947 Comparative studies of phagocytosis in normal and anemic blood Blood (Special Issue No 1) Morphol Hematol. 98
12 Berry L J & E C Haller 1947 The influence of anemia on phagocytic functions in rats Blood (Special Issue No 1) Morphol Hematol. 108
13 Berry L J R W Starr III & E C Haller 1949 The effect of surface active agents on phagocytos s J Bact 57 603
14 Matoth Y 1952 Phagocytic and ameboid activities of the leukocytes in the new born infant Pediatrics 9 748
15 Braude A I J Feltes & M Brooks 1953 Differences between the activity of mature granulocytes in leukemic and normal blood Proceedings of the Nati nal Meeting of the American Federation for Clinical Research Clin Res Proc 1 68
16 Mills C A 1949 Bone marrow nutrition in relation to the phagocytic activity of blood granulocytes Blood 4 150
17 Nungester W J & A M Ames 1948 The relationship between ascorbic acid and phagocytic activity J Infectious Diseases 83 50
18 Crepea S B G E Magnin & C V Seastone 1951 Effect of ACTH and cortisone on phagocytos s Proc Soc Exptl B ol Med 77 704
19 Tullis J L (Ed) 1953 Blood Cells and Plasma Proteins 272 Academic Press New York N Y
20 Zarafonetis C D R Harmon & P F Clark 1947 Influence of temperature upon opsonization and phagocytosis J Bact 53 343
21 Duchon L 1952 Une p euve du role cellulaire dans l étude du conflit cellules—micro-organismes Ind ce d activité cellulaire Ann inst Pasteur 82 254
22 Wood W B Jr & M R Smith 1949 The nature and biological s gnificance of the capsular slime layer of pneumococcus type III Trans Assoc Am Physicians 62 90
23 Wilson G S & A A Miles 1946 Topley & Wilson s Principles of Bacteriology and Immunity 1209 3rd ed Williams and Wilkins Baltimore Md
24 Knisely M H F H Bloch & L Warner 1948 Selective Phagocytosis I Microscopic observations concerning the regulation of the blood flow through the liver and other organs and the mechanism and rate of phagocytic removal of particles of the blood Kgl Danske Videnskab Selskab Biol Skrifter 4 1
25 Wright C S M C Dodd N G Brandt S M Elliott & J A Bass 1953 Erythrophagocytosis Standardization of a quantitative tissue culture test and its application to hemolytic malignant and infectious diseases J Lab Clin Med 41 169
26 Mabry D S J H Wallace M C Dodd & C S Wright 1953 Effect of specific antibody on the phagocytosis of virus modified RBC (Abstract) Proc Central Society for Clin Research 26 68 J Lab Clin Med 42 922
27 Wallace J H M C Dodd E Gardner Jr & C-S Wright 1954 Antigenic modifications of virus treated erythrocytes Federation Proc 13 516
28 Wright C S M C Dodd D S Mabry & J H Wallace To be published
29 Wood W B Jr M R Smith W D Perry & J W Berry 1951 Studies on the cellular immunology of acute bacteremia I Intravascular leucocytic reaction and surface phagocytosis J Exptl Med 94 521
30 Wood W B Jr 1953 Stud es on the cellular immunology of acute bacterial infections 87 The Harvey Lectures 1951-52 Academic Press New York N Y
31 Wood W B Jr 1951 The role of su f ce phagocytosis in acute bacterial infections 13 The 1950 Year Book of Pathology and Clinical Pathology H T Karsner & A H Sanford Eds The Year Book Publishers Chicago Ill
32 Von Moeschlin S W Zurukzoglu & J Crabbe 1953 Untersuchungen über den Einfluf von C rt son and ACTH auf d e Phagozytose de Leukozyten und Makrophagen Acta Haematol 9 277
33 Salis H 1948 Erythrophagocytosis in bone marrow culture with relation to antagonism of pteroylglutamic acid Proc Soc Exptl Biol Med 68 382

34 FENN W O 1921–22 Theoretical Response of Cells to Contact J Gen Physiol 4 384
✓35 NUNGESTER W J 1951 Mechanisms of man s resistance to infectious diseases Bact Revs 15 117
✓36 NUNGESTER W J & A M AMES 1952 Effect of selected chemical substances on phagocytosis J Infectious Diseases 90 51
37 NORTHROP J H & P H DE KRUIF 1921 The stability of bacterial suspensions III Agglutination in the presence of proteins normal serum and immune serum J Gen Physiol 4 655
38 WRIGHT C S W E HAINES & Q VAN WINKLE Microelectrophoretic studies of the normal and modified erythrocytes To be published
✓39 NUNGESTER W J A M AMES & W LANNING 1952 Electrophoresis studies of leukocytes and bacteria in relation to the mechanisms of phagocytosis J Infectious Diseases 90 61

LEUCOCYTES INVOLVED IN ANTIBODY FORMATION*

By Albert H. Coons, Elizabeth H. Leduc and Jeanne M. Connolly

Department of Bacteriology and Immunology and Department of Anatomy, Harvard Medical School, Boston, Mass.

The site of antibody production has intrigued and puzzled pathologists, hematologists, and immunologists since the discovery of antibodies in 1890 by von Behring. In recent years there has been a rising tide of experimental data bearing on this subject, both as to the organs involved in antibody production and as to the cell type responsible for the actual synthesis. These data have been extensively reviewed by Fagraeus,[1] by Burnet and Fenner,[2] and by McMaster,[3] so that fortunately it is not necessary to summarize this literature in detail.

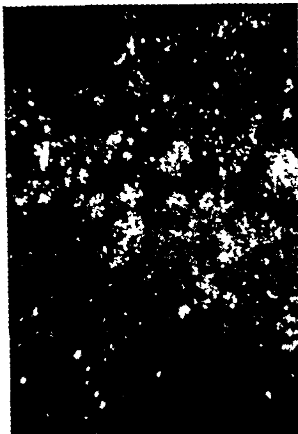
There have been three cell types on which suspicion in connection with antibody formation has been focussed: the macrophage, the lymphocyte, and the plasma cell. Evidence that the macrophage was involved in the synthesis of antibody was never direct and, indeed, all evidence on the subject is negative. For example, Ehrich, Harris, and Mertens[4] were unable to find antibody in macrophage-containing exudates at the height of antibody production, and Miller and Bale[5] have recently failed to find any incorporation of C^{14} labelled lysine into *gamma* globulin by the perfused rat liver, indicating that no *gamma globulin is formed in the liver, including of course the Kupffer cells.*

The evidence in favor of the lymphocyte is likewise circumstantial. It is based, we believe, on two misfortunes which could hardly have been anticipated by the investigators: (1) that in a lymph node stimulated by an antigen for the first time, considerably less than one per cent of the cells present are engaged in antibody production,[6] and (2) that the early forms of the cells which do form antibody are morphologically very similar to the early forms of lymphocytes. It may be, however, as Burnet and Fenner have suggested, that members of the lymphocyte series contribute to the long-continued formation of low levels of antibody, and there are hints in the evidence collected in our laboratory that this suggestion may be correct. However this may be, Harris, Rhoads, and Stokes could find no antibody in the thymus of immune rabbits.

On the other hand, the evidence for the participation of the plasma cell in antibody production has become, in our opinion, conclusive. Following the lead of Bing,[8] Bjørneboe and Gormsen,[9,10] Fagraeus,[1,11] Kolouch, Good, and Campbell,[2] Ehrich, Drabkin, and Forman,[12] Marshall and White,[14] Keuning and van der Slikke,[15] Moeschlin, Palaez, and Hugentobler,[16] Thorbeke and Keuning,[17] and Makinodan, Ruth, and Wolfe[19] all studied the cellular changes after repeated injections of antigen. In such animals there develop colonies of plasma cells in the spleen and lymph nodes containing several hundred cells per colony. These colonies develop in the red pulp of the spleen and in the medullary areas and around the lymphoid follicles of lymph nodes. These workers have shown that (1) the antibody content of such tissues is correlated

* Th[illegible] work reported here was supported by gr[illegible] from the Helen Hay Whitney Foundation [illegible] performed [illegible] by [illegible] of the authors (A. H. C.) [illegible] Investigatorship of the American Heart Association.

34 FENN W O 1921–22 Theoretical Response of Cells to Contact J Gen Physiol 4 384
√35 NUNGESTER W J 1951 Mechanisms of man s resistance to infectious diseases Bact Revs 15 117
√36 NUNGESTER W J & A M AMES 1952 Effect of selected chemical substances on phagocytosis J Infectious Diseases 90 51
37 NORTHROP J H & P H DE KRUIF 1921 The stability of bacterial suspensions III Agglutination in the presence of proteins normal serum and immune serum J Gen Physiol 4 655
38 WRIGHT C S W E HAINES & Q VAN WINKLE Microelectrophoretic studies of the normal and modified erythrocytes To be published
√39 NUNGESTER W J A M AMES & W LANNING 1952 Electrophoresis studies of leukocytes and bacteria in relation to the mechanisms of phagocytosis J Infectious Diseases 90 61



F   re 1   Rabbit   Low pow   ew f th   pl   n f a   bb t repeatedly i   j   t d w th hu a   gamma glob l n   od sa   ficed   th fifth d y   (th la t   j   tion   Se t   t est d with d l t h m   gamm   gl)] f ll wed by fl   sc   l belled   t body   Th   h te re   h w the fl o sc   ce   f th   lab lled   t bod   sc tt red   la d   f   t body   ont   g   lls   th   red pulp (ompa   w th F   greens figu e 6)

by the sudden dissolution of cells following another injection of antigen or of some other agent

Following a second injection of toxoid a month after the first the lymph node draining the site underwent a dramatic sequence of changes On the second day large cells with basophilic cytoplasm and large nuclei which were frequently in mitosis appeared in the medullary areas and around the periphery of lymphoid follicles These cells contained the first visible traces of antibody On succeeding days colonies of more mature cells containing more antibody in their cytoplasm and sometimes traces of it in their nuclei appeared in the same locations until by the eighth day there were colonies of plasma cells with large amounts of antibody (as judged by the increasing intensity of the fluorescence) in their cytoplasm The location and numbers of these cells at various stages in their development made it possible to correlate the presence of anti

with the presence of plasma cells, and (2) fragments of tissue containing plasma cells will continue to synthesize antibody when transferred to culture fluid *in vitro* The synthesis is more rapid if the tissue contains younger members of the plasma cell series especially large cells with basophilic cytoplasm called by Fagraeus "transitional cells" and by others[14, 15] plasmablasts or hemocytoblasts

The first direct demonstration of antibody in individual cells was carried out by Reiss Mertens and Ehrich[19] who showed that plasma cells from stimulated lymph nodes would specifically agglutinate the bacterial species which had been used as the stimulus Later, Hayes Dougherty, and Gebhardt[20] carried out similar experiments with cells from subcutaneous tissue where antigen had previously been deposited They identified the specifically agglutinating cells as lymphocytes However still later Moeschlin and Demiral[21] confirmed Reiss *et al*, using as an additional criterion for the identification of the cell type the presence of small cytoplasmic granules visible under the phase microscope which Moeschlin *et al*[16] had previously described as characteristic of plasma cells

In studies of antibody formation in our laboratory, use has been made of fluorescein labeled antibody as a histochemical reagent[22] This method allows the localization of antigen under the fluorescence microscope because of the specific precipitation of the labeled antibody over sites where the antigen is present If antibody containing sections are exposed to specific antigen *in vitro* the antigen fixed to the section by the presence of antibody can similarly be detected and thus by the use of two layers antibody can be localized in cells and parts of cells[23] When rabbits repeatedly injected intravenously with alum precipitated human *gamma* globulin or egg albumin were studied by means of this immunohistochemical reaction the islands of plasma cells in the spleens were found to contain unequivocal and perhaps large amounts of antibody in their cytoplasm and sometimes also in their nuclei (FIGURES 1 2) These cells were scattered in large aggregations throughout the red pulp In an occasional Malpighian corpuscle there were traces of antibody in some of the cells Similar groups of plasma cells were found in the medullary areas and around the lymphoid follicles of lymph nodes A few similar cells were encountered in the portal connective tissue of the liver along the basement membrane and in the stroma of the villi of the ileum[1] In brief in many of the areas where Bjørneboe and Gormsen[9] found plasma cells in their much more vigorously stimulated rabbits plasma cells containing antibody were identified

Studies were also carried out on the popliteal lymph node of the rabbit following the injection of diphtheria toxoid into the footpad Here after the first injection there were only occasional rather immature ("transitional" or "blast") cells in the medullary areas which were first demonstrable on the fourth day and increased somewhat in number up to the eighth day when they were mature The number of these cells was so small that their identification in adjacent sections was not possible This paucity of active cells probably accounts for the difficulties of identifying the cell type in animals which have received only one injection By the end of three weeks it was very difficult to find any cells It should be emphasized that there was no evidence in this material of storage of antibody in cells of any antibody which might be released

References

1 Fagraeus A 1948 Acta Path Microbiol Scand Suppl 204
2 Burnet F M & F Fenner 1949 The Production of Antibodies Macmillan Melbourne Australia
3 McMaster P D 1953 Sites of Antibody Formation in the Nature and Significance of the Antibody Response A M Pappenheimer Ed Columbia Univ Press New York N Y
4 Ehrich W E T N Harris & E Mertens 1946 J Exptl Med 83 373
5 Miller J J & W F Hale 1954 J Exptl Med 99 125
6 Leduc E H A H Coons & J M Connolly Studies on antibody production II The primary and secondary response in the popliteal lymph node of the rabbit In preparation
7 Harris T N J Rhoads & J Stokes 1948 J Immunol 58 27
8 Bing J 1940 Acta Med Scand 103 547
9 Bjørneboe M & H Gormsen 1943 Acta Path Microbiol Scand 20 649
10 Bjørneboe M H Gormsen & F Lundquist 194 J Immunol 55 121
11 Fagraeus A 1948 J Immunol 58 1
12 Kolouch F R A Good & B Campbell 1947 J Lab Clin Med 32 749
13 Ehrich W E D L Drabkin & C Forman 1949 J Exptl Med 90 157
14 Marshall A H F & R G White 1950 Brit J Exptl Path 31 157
15 Keuning F J & L B van der Slikke 1950 J Lab Clin Med 36 16
16 Moeschlin S J A Pelaez & F Hugentobler 1951 Schweiz med Wochschr 81 1247
17 Thorbecke G J & F J Keuning 1953 J Immunol 70 129
18 Makinodan T K F Ruth & H R Wolfe 1954 J Immunol 72 45
19 Rei F F Mertens & W E Ehrich 1950 Proc Soc Exptl Biol Med 74 732
20 Hayes S P T F Dougherty & L P Gebhardt 1951 Proc Soc Exptl Biol Med 76 460
21 Moeschlin S & B Demiral 1952 Klin Wochschr 30 82
22 Coons A H & M H Kaplan 1950 J Exptl Med 91 1
23 Coons A H E H Leduc & J M Connolly 1953 Federation Proc 12 439 also Studies of antibody production I A method for the histochemical demonstration of specific antibody and its application to a study of the hyperimmune rabbit In preparation



FIGURE 2 H gh pow f d p lp (mp re w th Keu g t al figure 3)

body in one section with the morphology of Giemsa stained adjacent sections[6, 23]

In some of the lymphoid follicles traces of antibody were discernible in some of the cells close to the center of the follicle In such areas it often appeared that there was antibody outside the cells as well Although the significance of this finding is not clear as yet it is consistent with the hypothesis that small amounts of antibody are also formed by lymphocytes Nevertheless by far the greatest amount of antibody was present in recognizable members of the plasma cell series Antibody was first found in cells at the blast stage and increased in amount in the cytoplasm as the cells multiplied and differentiated

It seems most likely in view of all the evidence now available that the plasma cell differentiates specifically in response to antigenic stimulation Moreover the initial antigenic stimulus has a hidden effect which does not become manifest until it is unveiled by the burst of growth and protein synthesis which follows a second specific stimulus

ation one must not overlook that any inference drawn is only one of analogy Until leukotaxine is purified further and until its structure is known it is important to concentrate closely on its extraction from exudates

Duthie and Chain have likewise obtained a biologically active polypeptide from the peptic hydrolysate of blood fibrin which was capable of increasing capillary permeability and of inducing leukocytic migration [8] Cullumbine and Rydon who also confirmed the presence of leukotaxine in goat exudates were likewise capable of obtaining active polypeptides by enzymatic digestion [9] These investigators showed that various other irritants such as mustard gas or lewisite, and the edema fluid of lungs exposed to phosgene were all able to increase capillary permeability and to induce leukocytic migration The effect was shown to occur even in man by application of mustard gas or by the intra dermal injection of leukotaxine followed by the intravenous injection of Evans blue [9] Cullumbine and Rydon made the very interesting observation that leukotaxine was destroyed or inactivated by normal blood plasma or serum but failed to be inactivated by the sera from animals suffering from shock They were also unable to recover leukotaxine in the sera of shocked animals [9]

Spector has recently extended the studies of these investigators on peptides and on the relation of peptides to inflammation [10] His attention was primarily directed to a study of the peptic digest of fibrin The peptides obtained were capable of increasing capillary permeability of inducing leukocytic migration and of causing swelling of endothelial cells By treating the digest with ammonium sulfate at 75 per cent saturation Spector succeeded in dissociating two fractions The resulting precipitate contained the three biological properties of the original digest *i e* permeability chemotactic and endothelial cell swelling capacity On the other hand the supernatant fraction had no permeability property displaying only leukocytic migratory and endothelial cell swelling abilities The average length of the peptide chains was different in both fractions of the digest after $(NH_4)_2SO_4$ precipitation In the precipitate fraction at an isoelectric point at pH 3 8 there were 14 amino acids whereas in the supernatant the peptide contained only five amino acids Spector was thus able to separate the chemotactic factor in the fibrin digest from the permeability factor by obtaining peptides of different chain lengths To the writer it is of interest that the ammonium sulfate supernatant fraction of the digest devoid of any permeability capacity failed by chromatographic study to show any proline or amino butyric spots These spots were present in the other fractions of the digest The role of proline or amino butyric in the mechanism of inducing increased capillary permeability by the digest of fibrin should perhaps be determined In his study of inflammatory exudates however Spector was incapable of bringing about any such dissociation [10] In the rabbit guinea pig and goat exudate this investigator by using either 50 or 75 per cent $(NH_4)_2SO_4$ obtained peptide fractions containing both permeability and chemotactic properties In 1938 the writer stated in his original description of the two biological properties of leukotaxine The analytical procedure has thus failed to dissociate the two factors Although this type of evidence is strongly suggestive of a single substance it is however to be borne in mind that only the certitude of complete purity of the material can establish this as a definite fact [3] Thus

FACTORS CONCERNED IN THE MOBILIZATION OF LEUKOCYTES IN INFLAMMATION*

By Valy Menkin

The Agnes Barr Chase Foundation for Cancer Research Temple University School of Medicine Philadelphia Pa

In this survey of some of the factors concerned in the mobilization of leukocytes in inflammation I propose to discuss three topics (1) the present status of leukotaxine, (2) the cytological sequence in an acute inflammation in relation to the hydrogen ion concentration and (3) the factors that determine the leucocyte level in the circulating blood with a concomitant acute inflammation

1 The Present Status of Leukotaxine and the Local Migration of Polymorphonuclear Leukocytes in Inflammation

About 18 years have elapsed since the first description of leukotaxine as a diffusible factor from whole or cell free exudate [1] This factor was subsequently isolated and crystallized [2] The substance was found both to increase capillary permeability at the point of its injection in tissue and to induce the local migration of polymorphonuclear leukocytes [2,3] The chemotactic property of leukotaxine was also demonstrated by *in vitro* studies [4] By deproteinization with either pyridine or dioxan and acetone followed by butanol treatment a final product was obtained apparently devoid of gross impurities since it could even be brought to the crystalline state Morimoto in Japan has recently obtained and described the same needlelike crystals of leukotaxine as occasionally obtained by the writer [2,5] Now the whole exudate contains both biological properties of increasing capillary permeability and of inducing leukocytic migration The final crystalline product possesses the same two biological properties In all of the studies of the writer on the chemical identification of various substances from inflammatory exudates the exudate as such serves first as a base level This base precludes to a large extent the possibility of a chemical artefact The utilization of various simple tests for protein and amino groups combined with determinations of the amino nitrogen before and after hydrolysis led us to the view that leukotaxine was a relatively simple polypeptide to which there may possibly be attached an as yet undetermined prosthetic group [6] At about the same time it was observed that treatment of either blood serum or crystalline serum albumin with crystalline trypsin resulted in the formation of intermediary protein breakdown products that had both permeability and chemotactic properties [7] This finding was merely suggestive and it added further support to the concept of leukotaxine as a polypeptide I should like to stress here that such enzymatic products may resemble leukotaxine but should not be considered to be leukotaxine Leukotaxine is derived from exudative material and reasonably explains the mechanism of two of the basic sequences of inflammation Breakdown products of proteins obtained by enzymatic digestion are merely suggestive but in their final evalu

* Th work r po t d th p pe w I d by g t f n th U t I St t P bl He lth Serv (C 3778) and by a gra t f om th D t A W d Soc ct A ym B w t I d
Th s paper is N 49 f a se t t I d St d o I fl mm t o

taxinelike material by utilizing similar extraction procedures as employed for exudates[3] (see Menkin[2] p 146 footnote)

(4) Cullumbine and Rydon had used turpentine on the goat and also other unrelated irritants such as mustard gas and lewisite the blister fluid from heated areas and lung edema fluid following phosgene administration[9] The writer had used besides turpentine the effects of burns an aleuronat starch mixture in rabbits croton oil in olive oil and human exudative material[2 4] Recently by merely crushing and mincing muscle tissue of the rabbit a leukotaxinelike material was obtained from the extract[13] Thus an irritant was found to be unnecessary but damage to cells seemed to be a requisite in order to obtain leukotaxine or at least a leukotaxinelike material having the two biological properties described above

The foregoing facts in addition to the controls used when turpentine was utilized and extracted *per se* and the inability of normal blood serum to yield any appreciable amounts of leukotaxine when similar chemical procedures were employed render Harris s viewpoint somewhat difficult to accept As pointed out above it is conceivable that the discrepancies between the two laboratories may be partly referable to the utilization of different animals and also to the unequal sized thoracic cavities of the latter When attempts are made to substantiate the studies of other investigators it is well at first to use the same techniques on the same animal before deviating to other procedures however important

In a recent review Harris argues without supplying any new factual information or evidence that leukotaxine has not been convincingly established as a chemical entity[14] Assertions as such have to be backed by additional facts and not merely by arguments Let us examine the essence of Harris s contention and inferences

(1) On page 547 of the review in question Harris admits that there is little doubt that the material extracted by Menkin from inflammatory exudates does increase capillary permeability and cause emigration of leukocytes from vessels when it is injected into the tissues These are precisely the properties of the whole untreated exudate as the present writer has often pointed out Menkin has demonstrated that exudative material has the biological properties of increasing capillary permeability and of inducing leukocytic migration and that exactly the same effect is duplicated by the extracted material or leukotaxine Harris implies that this is a nonspecific reaction elicited by any polypeptide that irritates blood vessels This implication is not quite factual for as it has been pointed out other peptides such as glutathione and a great number of amino acids induce no such reaction (see Menkin[4] p 47 footnote) ACTH (corticotropin) which is presumably a mixture of peptides fails to induce any increase in capillary permeability Furthermore and most important other peptides extracted from exudates such as for instance exudin * fail to induce both combined properties of increasing capillary permeability and of causing the the migration of leukocytes Accordingly there seems to exist a biological spec

* P l m ry h m t g ph t d d t th t d seem t be lo g h pept d (r bl h d bsers t by V K l)

far, although there may be two separate substances in leukotaxine, such a dissociation has not been accomplished In brief Spector has succeeded in obtaining a separation in the digest of fibrin but he has not been able to report any such dissociation in inflammatory exudates[10] The writer has recently pointed out that the chemotactic property of leukotaxine when kept standing for months frequently becomes obliterated while the permeability property persists[11] Such observations do not represent a dissociation but may simply indicate the greater instability of the chemotactic group in the leukotaxine molecule A difference in the threshold of the two biological properties had already been noted previously[3] In summary as matters stand at present we still have to consider leukotaxine as a single entity The difference in the results obtained by Spector in the fibrin digest from those observed in inflammatory exudates[10] again emphasizes the necessity of concentrating our attention on studying at least at present leukotaxine as extracted only from inflammatory exudates rather than drawing inference from enzymatic digests of proteins

Harris has recently described his findings on the chemotactic activity of leukocytes *in vitro* by a new technique[12] His observations seem to indicate that chemotaxis as studied *in vitro* is a different phenomenon from what is termed chemotaxis *in vivo* It is quite possible that the complex *in vivo* phenomenon as observed in inflammation is the resultant of a number of factors whereas the *in vitro* results represent the behavior of a single factor This possibility may well account for the discrepancies observed by Harris in a number of microorganisms

Harris in his studies on exudates has utilized the rabbit's pleural cavity following the introduction of 1 ml of turpentine He reports that the resulting rabbit exudate from this dose of turpentine is toxic to leukocytes by *in vitro* studies[12] He points out that turpentine *per se* is toxic to these cells By the procedure utilized for leukotaxine extraction Harris has recovered nonvolatile derivatives of turpentine The results obtained by the writer[3] in 1938 and therefore some of his interpretations are at variance with the views of Harris[12] These differences can be listed briefly as follows

(1) Harris has used the pleural cavity of the rabbit whereas the writer has utilized the dog s pleural cavity with the same type of irritant The size of the pleural cavity of the two animals should be considered in estimating the toxicity and the dilution effect of about the same dose of turpentine on the effect on leukocytes A possible differential susceptibility to injury on the part of leukocytes in the two animals treated with the same irritant should also be kept in mind

(2) The writer found that a very large number of leukocytes are viable in the first day of inflammation following the introduction of 1 to 1 5 ml of turpentine in the pleural cavity of the dog This response was shown both by the usual criteria of fixed stained preparations and by supravital staining [in this connection *cf* Figure 1 in Menkin and Warner[31] (1937) and the footnote p 28 of the same paper See also Menkin[17] (1939)]

(3) Turpentine was shown by the writer to contain no leukotaxine or leuko

taxinelike material by utilizing similar extraction procedures as employed for exudates[2] (see Menkin[8] p 146 footnote)

(4) Cullumbine and Rydon had used turpentine on the goat and also other unrelated irritants such as mustard gas and lewisite the blister fluid from heated areas and lung edema fluid following phosgene administration[9] The writer had used besides turpentine the effects of burns an aleuronat starch mixture in rabbits croton oil in olive oil and human exudative material [4] Recently by merely crushing and mincing muscle tissue of the rabbit a leukotaxinelike material was obtained from the extract[13] Thus an irritant was found to be unnecessary but damage to cells seemed to be a requisite in order to obtain leukotaxine or at least a leukotaxinelike material having the two biological properties described above

The foregoing facts in addition to the controls used when turpentine was utilized and extracted *per se* and the inability of normal blood serum to yield any appreciable amounts of leukotaxine when similar chemical procedures were employed render Harris s viewpoint somewhat difficult to accept As pointed out above it is conceivable that the discrepancies between the two laboratories may be partly referable to the utilization of different animals and also to the unequal sized thoracic cavities of the latter When attempts are made to substantiate the studies of other investigators it is well at first to use the same techniques on the same animal before deviating to other procedures however important

In a recent review Harris argues without supplying any new factual information or evidence that leukotaxine has not been convincingly established as a chemical entity[14] Assertions as such have to be backed by additional facts and not merely by arguments Let us examine the essence of Harris s contention and inferences

(1) On page 542 of the review in question Harris admits that there is little doubt that the material extracted by Menkin from inflammatory exudates does increase capillary permeability and cause emigration of leukocytes from vessels when it is injected into the tissues These are precisely the properties of the whole untreated exudate as the present writer has often pointed out Menkin has demonstrated that exudative material has the biological properties of increasing capillary permeability and of inducing leukocytic migration and that exactly the same effect is duplicated by the extracted material or leukotaxine Harris implies that this is a nonspecific reaction elicited by any polypeptide that irritates blood vessels This implication is not quite factual for as it has been pointed out other peptides such as glutathione and a great number of amino acids induce no such reaction (see Menkin[4] p 47 footnote) ACTH (corticotropin) which is presumably a mixture of peptides fails to induce any increase in capillary permeability Furthermore and most important other peptides extracted from exudates such as for instance exudin * fail to induce both combined properties of increasing capillary permeability and of causing the the migration of leukocytes Accordingly there seems to exist a biological spec

* Prel m ry h m t g phi t d s d t th t xud seem t be l g h pept d (p bl h d bsers t by \ kaln)

ificity in leukotaxine not elicited by all other peptides of the exudate Of course, as stated previously, the discovery of leukotaxine does not, however, preclude the possibility that other active substances exist in the exudate, but these substances have not yet been identified and differentiated (at least with any degree of certitude), whereas the presence of leukotaxine has been demonstrated (see Menkin[4] p 42)

(2) Harris comments on the question of the 'relatively pure' state of leukotaxine Leukotaxine has always been considered to be purified material extracted from exudate,[2, 8] and even crystallization is no criterion for purity, as is well known Different methods of chemical extraction from exudate have yielded, over the years essentially the same type of material with the same biological properties[11, 15]

The recent use of N acetic acid in the final precipitation from the acetone supernatant residue probably removes any lipids as impurities in the precipitate[11, 15] On the other hand even in 1938, the original paper of the writer stressed the necessity of complete purity of the material (Menkin[2] p 149)

Leukotaxine is the material purified from the crude exudate but its absolute purity has never been claimed and, in fact the necessity of further chemical purification has often been emphasized by the writer (Menkin[16] p 135, Menkin[13] Menkin[4] p 147)

In *Nature* the writer states in regard to the various factors isolated from exudates These substances are at present relatively crude and it is hoped that they will be purified further[17] Chemical purity however, is not to be confused with chemical entity as Harris seems to imply Many specific biological substances are known to exist without having as yet been completely purified ACTH (as it is distributed) seems to be one such substance Yet no one would question its existence The specific biological effect of leukotaxine has been amply confirmed Harris seems to take no cognizance of the confirmation by other investigators of this biologically active substance Such investigators include among others who have studied this significant substance, Cullumbine and Rigdon[9] Morimoto[5] and Pasquali[15]

It is significant to point out also that as a control when blood serum was extracted for leukotaxine essentially no activity resulted in the end product Yet the same original chemical procedure was utilized as in the case of the inflammatory exudates (Menkin[13] p 69 footnote 12 and Menkin[9])

(3) As pointed out above an irritant is not even a necessity mere cellular injury seems to be requisite This fact considerably weakens the criticism that possibly the irritant exists in the end product *i e* in leukotaxine Leukotaxine or leukotaxinelike material has been extracted after severely injuring muscle tissue[13]

(4) The biological activity of leukotaxine is specifically abolished or at least markedly reduced by adrenal cortical extract cortisone or Compound F[4, 11] On the other hand the activity of exudin also derived from exudate is not altered by the above corticosteroids but only by ACTH[3] Both leukotaxine and exudin increase capillary permeability although exudin fails to induce appreciable emigration of leukocytes These facts support the idea that leukotaxine is a rather specific chemical and biological entity in exudates

Harris in his review completely ignores these significant differential observations In some recent as yet unpublished studies it has been found by the writer that whereas leukotaxine increases the permeability of sea urchin ova to water [1] exudin another apparent intermediary product of protein breakdown extracted from acid exudates and capable of increasing capillary permeability fails to increase the permeability of the same sea urchin ova (*Arbacia punctulata*) to water It is conceivable in view of these facts that possibly leukotaxine acts on the endothelial cell permeability whereas exudin might perhaps act on the intercellular cement substance in capillaries At any rate the significant point is that various intermediary breakdown products from exudates have different but specific biological effects

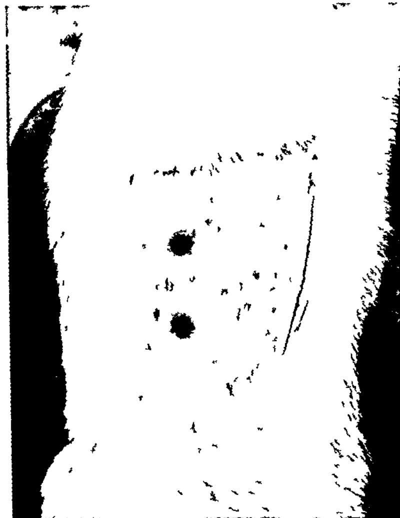
(5) Is leukotaxine chemotactic *per se*? Without any evidence of his own or or that of any other investigator Harris dismisses this point as unproved despite the earlier facts presented by the writer In 1938 leukotaxine was shown to be chemotactic in two ways[3]

(a) By placing it in capillary tubes sealing one end of each tube and introducing the tubes under ether anesthesia in the peritoneal cavity of rabbits with inflammation induced by aleuronat and starch These experiments were controlled by using also the inactive material obtained from blood serum by the same methods utilized for the extraction of leukotaxine from exudates Saline distilled water and lactic acid were also employed as additional controls The migration of leukocytes in the leukotaxine tubes was much more striking and further inward than in the control tubes Harris objects to the capillary tube method in view of Pfoehl s work [25] Pfoehl was skeptical of the capillary tube method for it implied that the leukocytes locomote against gravity He was of the belief that the leukocytes were swept in the tubes by convection currents at the mouth of the tubes Further observations *in vivo* and *in vitro* by Ruchladew seem to support Pfoehl s point of view [26] Although it is readily admitted that more modern *in vitro* methods as used by McCutcheon[27] and more recently by Harris[12] are more precise than the older capillary tube techniques this fact does not necessarily mean that all observations made with the capillary tube technique especially when well controlled should be discarded The writer has controlled his observations very carefully with leukotaxine and the material extracted from blood serum which in turn is inactive and has found a definite difference in migration when the capillary tubes were introduced in the inflamed peritoneal cavity A similar difference was found with the use of saline or distilled water or lactic acid If the effects obtained were entirely due to convection currents or even osmotic pressure difference it is somewhat difficult to see how the tubes enclosed in the peritoneal cavity would show such sharp differences in reaction It is an assumption to assert that convection currents even if such currents were set up in the peritoneal cavity (for which there is as yet no proof) would account for the difference between leukotaxine from exudate and similar but inactive material from blood serum The inferences drawn by Harris are unwarranted by the observed facts

(b) The writer has pointed out that particles of leukotaxine on a slide induce in a relatively short time the clustering and collecting of leukocytes around it This clustering fails to occur when as a control either charcoal or reduced

iron powder are utilized (see Menkin,[4] Figures on pp 54 and 55) This observation namely, that leukotaxine is *per se* chemotactic was considered to be supporting evidence to the capillary tube observations Harris on the other hand takes exception to this interpretation In the first place he makes no comments about the capillary tube observations, but since throughout his review, he rejects information obtained by this capillary technique he evidently dismisses also such observations by the writer without any further remarks In regard to the *in vitro* observations of the clustering of supravitally stained leukocytes around particles of leukotaxine he expresses the belief that the cells are dead, since the cells appear rounded with visible nuclear membranes If the cells are dead, how they were able first to locomote and then to gather around the particle of leukotaxine is a complete enigma to the writer The same cells when observed on a slide with charcoal or reduced iron powder failed to display any adherence to this type of material (Menkin[4] *cf* Figures 15 and 16 pp 54 and 55) Furthermore, if the cells are dead the nuclei would tend to take the neutral red stain (*cf* for instance in this connection Figure 26 p 84 Menkin[4] for the nuclear neutral staining when cells are either markedly injured or dead) It is true that as supravitally stained leukocytes approach nearer to concentrated particles of leukotaxine, they often do not contain any stained granules in their cytoplasm The exact meaning of this observation is not as yet quite clear On the other hand if one examines the slide at a distance from leukotaxine the granules are stained supravitally in the cytoplasm of many of the leukocytes The tendency for the cells to appear round is possibly referable to the fact that the cells on the slides when drawn by a well known and accurate artist at the Harvard Medical School (E Piotti) had been removed from the temperature of 37° C in the incubator and were drawn at room temperature of approximately 20 C or so

However in view of Harris s comments this question was again reinvestigated with two different fractions of leukotaxine namely the evaporated acetone supernatant fraction after deproteinization and the N acetic fraction of leukotaxine recently described[11 15] Essentially the same results as originally described were obtained A total of seven separate experiments was performed Leukotaxine was placed on a supravitally prepared slide with neutral red A drop or two of a one day old exudate was added fairly near to the particle of leukotaxine The exudate was derived by the injection under Nembutal anesthesia of either 1 5 ml of turpentine into the right pleural cavity of dogs or by the introduction of 0 5 ml of 5 per cent croton oil in olive oil A cover slip was adjusted and then rimmed with a molten mixture of paraffin and vaseline The cells were studied in a warm box at 37 C In brief it was found that in the first 10 to 15 minutes there was no particular aggregation of leukocytes around the particles of leukotaxine There seemed however to be an orientation in the direction of that material which became much clearer within a period of from 40 minutes to one hour This orientation is illustrated in FIGURE 2 Definite ameboid polarization towards the particles of leukotaxine was observed Furthermore in that interval there was already an appreciable number of leukocytes adhering to the leukotaxine It would be well to investigate also whether leukotaxine has properties of stickiness The present



F[illegible] 1 Th[illegible] capillary permeability [illegible] treat[illegible] [illegible] followed by the [illegible] to the [illegible] Leukota[illegible] [illegible] subsequently trypan blue wa[illegible] accumulation [illegible] to the [illegible] capillary perm[illegible]

observations in addition to the earlier ones[4] suggest this possibility After one to three hours the clustering of leukocytes around the particles of leukotaxine becomes even more striking On the other hand when particles of charcoal were utilized as control there was no aggregation collection or stickiness of the leukocytes around this carbon material Observations with the charcoal were also extended for a period of about three hours Sometimes

granules of charcoal were found after prolonged intervals to be phagocyted within the leukocytes, but at no time was aggregation of cells observed around the particles of charcoal After many hours at times small clusters of leuko cytes were observed in the control slides these clusters, however were not spatially referable to the carbon particles This phenomenon was interpreted although the interpretation was not proved as the effect of irregular concen tration and precipitation from the exudate of leukotaxinelike material with a subsequent attraction of leukocytes around it The effect however may be a leukergylike phenomenon as described by Fleck [8]

When as control blood serum was extracted for leukotaxine and similar ob

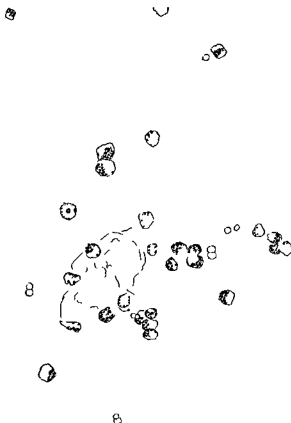


FIGURE Th t h tat ff t f l k t l k t D w g h w g th g f s p tally t d l kocyt t w d y t l s f l k t (p p d b N a t l m th i N t th t mb f l k yt l d l t d dh t t th l k ta Th se ll d t fi d by th g lat b t th cyt l m g l t ta l Th gg t f t k t th le k ta m t l Th g g t l ge f l kocyt th d w g f m bo t 40 m t t b t 1 h d 15 m t ft th b g g f th p m t Th y t t t figu 35 Dy m f f ft mm t 1940 p 54 wh th t l w s m h t l g m ly b t 3 h d th l t g f ll w m h m m k d Also t th t d ff t f t f l k t i e w s t l zed t th t m Cont l bserv t w th h l d d th i t t g n r ggregat f l k cyt w th th sam lo g r t l

servations were carried out *in vitro* no evidence of any chemotactic response was noted Such material from serum was found completely inactive by utilizing both *in vitro* and *in vivo* methods

Several experiments were also performed to test the effect of leukotaxine on the chemotactic activity of mononuclear phagocytes Harris points out that his investigations have indicated that monocytes were found to react chemotactically and perhaps even more directly, to the same stimuli as polymorphonuclear leukocytes [14] It therefore became of interest to investigate the *in vitro* response to leukotaxine of mononuclear phagocytes Seven distinct experiments were again conducted utilizing two-day-old to four-day-old exudative material Such examples were found to be at an acid pH (usually slightly above 6 0) and to have about two thirds of the cell content consisting of apparently viable mononuclear cells The *in vitro* chemotactic studies were carried out as described above for polymorphonuclears It was found that the mononuclear cells seemed to react precisely in the same way as polymorphonuclear leukocytes Harris had pointed out that these cells do not move as rapidly as polymorphonuclears [14] After several hours essentially all of the leukocytes of the sample of acid exudate tested had gradually converged and had adhered as a moderately thick layer to the leukotaxine This action stood in sharp contrast to the absence of any such orientation by these cells towards the carbon particles

These observations may have definite implications if substantiated by *in vivo* studies Since the mononuclear leukocytes move less rapidly than the polymorphonuclear leukocytes [14] it is conceivable that the early location of the mononuclears at the periphery of an inflamed focus may be referable in part to the same chemotactic stimulus namely leukotaxine The differential rate in respective mobility of the leukocytes involved could possibly account for the primary peripheral location of the mononuclear phagocytes With the subsequent development of a local acid pH [29, 30] and the consequent inability of the polymorphonuclears to survive a gradual replacement at the site of inflammation by mononuclear phagocytes would ensue

In conclusion the same type of observations as have been made previously but more detailed again substantiated the fact that leukotaxine is *in vitro per se* a chemotactic substance This substance seems to attract both polymorphonuclears and mononuclear phagocytes The implication of these findings in regard to the spatial position of these respective cells in an inflamed area is discussed

Finally it would be desirable for Harris to supply more precise and more substantiating facts in regard to his views of the absence of chemotaxis *in vivo* His interpretation concerning the inability of proteolysis to occur within several minutes following a relatively mild degree of cellular injury also should be supported by stronger evidence Since the required degree and rate of proteolysis by injured cells for the production of leukotaxine are unknown the inferences drawn by Harris seem wholly conjectural to the writer

Is there only one substance in inflammatory exudates capable of explaining the increased capillary permeability and the leukocytic migration in inflammation? As stated previously the recovery of leukotaxine from exudate does not

preclude the possibility that other biologically active substances may be present in the exudates [4] The contention of Moon and Tershakovec that numerous protein split products are able to increase capillary permeability and induce migration of leukocytes[31, 3] is no evidence that leukotaxine and for that matter the other chemical factors may not be the primary substances concerned in the reaction The stereopatterned reaction of increased capillary permeability and leukocytic migration is conceivably referable primarily to cell injury by the irritant whatever its nature with the consequent liberation of leukotaxine from the injured cell It is quite possible that the irritant may also contain leukotaxinelike material This possibility for instance has been pointed out in the case of *Staphylococcus aureus* and it may partly account for the intense tissue infiltration induced by this microorganism [4] but this synergism is unessential in our understanding of the release from the injured cell of a common denominator namely leukotaxine [13] Some degree of cellular injury is required to obtain leukotaxine whether obtained in an inflamed area or by mechanical means [4, 13] Minimal autolysis involves some amount of cellular injury This fact renders the thesis of Moon and of his collaborators difficult to accept namely that a chemotactic agent is found in normal tissue [33] Unhemolyzed blood serum has not yielded any appreciable quantity of leukotaxine at least by the methods employed for its detection and extraction [19, 20] The extent of cellular injury or degradation required before leukotaxine is released is unknown Quite obviously leukotaxine must have some as yet unknown precursors in the normal cell but leukotaxine as now obtained requires first some degree of cellular injury

Histamine has been shown in many earlier publications not to be leukotaxine and the evidence will therefore not be reviewed here [4, 30, 31] There is also no definite evidence that histamine liberates leukotaxine Histamine, for instance does not increase cell permeability of sea urchin ova whereas leukotaxine increases directly the permeability to sea water of such ova by over 50 per cent [4] The writer is informed that Rocha e Silva has recently stated that depletion of histamine by 48/80 has failed to decrease the inflammatory response [34] With this histamine depletor however Miles and Miles have made observations which lead them to believe that leukotaxine increases capillary permeability by liberating histamine [35]

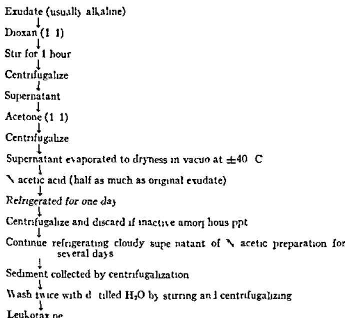
Feldberg also utilizing 48/80 arrives at the conclusion that histamine plays a role in the increased capillary permeability due to inflammation [36] It would be of interest to determine whether 48/80 depletes histamine without injuring the cell and whether or not it releases leukotaxine * Biozzi Meni and Ovary have studied the changes produced by leukotaxine and histamine on the skin of albino rats By the use of small doses of antihistaminic drugs they have concluded that leukotaxine acts without the intervention of any histaminic mechanism [35]

The writer has concerned himself of late in obtaining active leukotaxine from exudates by a simplified method which has recently been described (SCHEME 1) [11, 15]

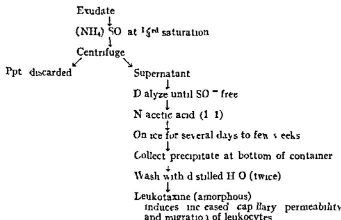
* [illegible] rythem [illegible] path [illegible] [37]

More recently in still preliminary work we have utilized the earlier observation that leukotaxine is carried along with the precipitate obtained by treatment of the exudate at one half saturation of ammonium sulfate (SCHEME 2) [1] The euglobin from an exudate at an alkaline pH is disposed of by precipitation *with ammonium sulfate at one third saturation The supernatant containing*

SCHEME 1 FOR EXTRACTION OF LEUKOTAXINE



SCHEME 2 FOR EXTRACTION OF LEUKOTAXINE



the pseudoglobulin albumin fraction (*i e* the alpha and beta globulins plus the albumin), after removal of the sulfate ions is then treated with an equal volume of N acetic acid and refrigerated After a period of from a few days to several weeks or even months an amorphous material separates out, which is very powerful in inducing both an increase in capillary permeability and leukocytic migration (FIGURES 1 and 3) In this connection it is of interest that Pashkina, in the Soviet Union, has recently substantiated the original observation of the writer that experimental and human exudates increase capillary permeability to a dye and cause leukocytic migration [39] Pashkina states that these properties of exudates are connected with a globulin fraction (probably the beta globulins) Although leukotaxine is recovered as a diffusible factor from whole exudate it has been pointed out previously that the purified fractions, probably due to their combination with the extractive chemicals or due to their insolubility in water tend to be frequently indiffusible [4] At present we are engaged in studying the chemical and physical properties of the various fractions of leukotaxine and in comparing them with each other We are also trying to separate further from a given sample of exudate leukotaxine and the other permeability factor termed *exudin* This latter factor however is not chemotactic and tends to be more prevalent in acid exudates [3]

In a recent unpublished study utilizing paper partition chromatography my associate Doctor W Kalnins has found that leukotaxine obtained by the method referred to above[11, 15] yields five distinct amino acids namely leucine valine alpha alanine glutathionine and glycine Aspartic acid and glutamic acid are also present but these two amino acids appear as a streak on the chromatogram and therefore as yet have not been readily dissociated These studies add further support to the view that leukotaxine is a relatively simple polypeptide

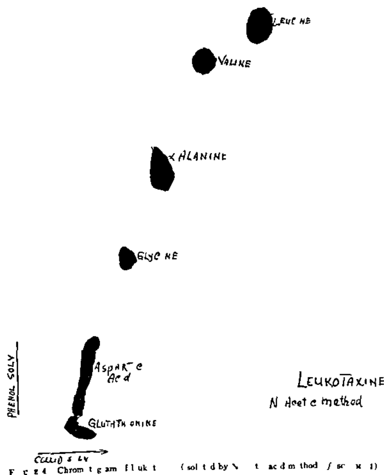
This discussion on leukotaxine cannot very well be terminated without a few words about the anti inflammatory effects of steroids on leukotaxine As you recall the extract of the whole adrenal cortex extract and cortisone or Compound E were first shown in 1940 and 1942 [1] – to have this property * A convenient index of cellular activity can be obtained on sea urchin ova by studying the incidence of cleavage in ova exposed to cortisone hydrocortisone (Compound F) or ACTH prior to their fertilization [41] The corticosteroids and ACTH were specifically found to suppress the cellular activity of these invertebrate ova as gauged by the incidence of cleavage [41] These findings on cellular activity suggested to us a study of the anti inflammatory mechanism The writer had demonstrated that the injured cell liberates a number of chemical factors which reasonably explain the biological manifestations of inflammation [16] One

I re t p pe Moo a d T h k c h t lly fi m d th g l t d f th w t r [38] These i est g to h w h som what m q t d th w t t t g th t M k p t d th t co t sone ac t t lo inj t d t d rm lly i d j d d t t se p ll ry pe m b l ty as how by the trypa bl t t [39] Th t l bse t po t d by M k p 2/9 w tat d foll ws Comm rc l t so t t pe (Compo d F) f th dr l t d p d i pill ry perm b l ty Th re sed cap llary perm b l ty d d doe t se m t be f bl t ort so e b t ather t th h l in wh h t so tat pe d l C t so powd f l t se cap llary perme b lity Th se f ts th w a t ly d ff t l ght th q t t f Moo d T h ko ec nd th terpretat they seem to g Th w t r h g l t l f 1942 [38] h h w that co t so (Compound E) p d ses cap llary perm b l ty wh f t re sed by th l k ta o by a alk lin exudat [28, 38]



F 3 Th ff t f th l k t f t j t d t th tan f ur l th d ped f l kocyt l h d 10 m t f llow g th t t oculat f that l k t f t (se d sch m f t act f l k ta employ d) ×25

of these factors is leukotaxine Would the injured cell in an inflamed area when exposed to an anti inflammatory steroid, be likewise suppressed in its activity so that it becomes unable to form some or all of these chemical factors? Repeated injections of 10 to 20 mgm of Compound F into an acutely inflamed pleural cavity in the dog represses the formation of any active leukotaxine, both in regard to its property of increasing capillary permeability (*cf* Figure 9, p 140 of Menkin[15]) and of inducing the diapedesis of polymorphonuclear leukocytes (FIGURES 5, 6)[4] The same repressive tendency was found to occur with some of the other factors, also in some preliminary experiments with the repeated local administration in the inflamed area of cortisone and ACTH These latter studies are being pursued further It thus seems as if the anti inflammatory mechanism of corticosteroids is primarily at a cellular level and is referable to a suppression of the very chemical factors concerned in inflammation such as for instance leukotaxine[4] The exact mechanism of this suppression of cellular activity is unknown In this connection it is conceivable that

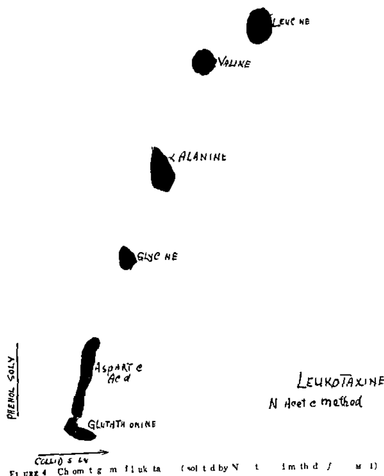


F c e 4 Chrom t g am f l uk t (sol t d by \ t ac d m thod / sc x 1)



F [illegible] 5 Eff [illegible] th [illegible] k of [illegible] bb t f le k t [illegible] d [illegible] g m gi t [illegible] f polymo phou l a le kocyt bou [illegible] ho [illegible] bseq t t th [illegible] f the sub ta ce [illegible] c d [illegible] t ro f om a sampl f ca a [illegible] date Th dog had re [illegible] ed [illegible] je tio f t rpentin into th [illegible] ght pleu l a ty f llow d by tw [illegible] ce s da ly in) tio f salin [illegible] h 3 c X400

of these factors is leukotaxine Would the injured cell in an inflamed area when exposed to an anti inflammatory steroid be likewise suppressed in its activity so that it becomes unable to form some or all of these chemical factors? Repeated injections of 10 to 20 mgm of Compound F into an acutely inflamed pleural cavity in the dog represses the formation of any active leukotaxine both in regard to its property of increasing capillary permeability (*cf* Figure 9, p 140, of Menkin[15]) and of inducing the diapedesis of polymorphonuclear leukocytes (FIGURES 5 6)[42] The same repressive tendency was found to occur with some of the other factors also in some preliminary experiments with the repeated local administration in the inflamed area of cortisone and ACTH These latter studies are being pursued further It thus seems as if the anti inflammatory mechanism of corticosteroids is primarily at a cellular level and is referable to a suppression of the very chemical factors concerned in inflammation such as for instance leukotaxine[4] The exact mechanism of this suppression of cellular activity is unknown In this connection it is conceivable that



Fi ure 4 Ch om t g m f l uk ta (sol t d by N t l m th d / M l)

the basic work of Clark[43] who recently showed that cortisone interferes with protein synthesis may prove significant in our further understanding of the mechanism involved in peptide formation by the injured cell *

2 The Cytological Picture in Inflammation

The polymorphonuclear leukocytes are usually the initial cells at the beginning of a developing inflammatory reaction These cells are followed by the mononuclear phagocytes (macrophages) This cytological sequence was first pointed out by Borrel in 1893[45] This sequential order seems referable to the developing acidosis in the acutely inflamed area[29, 4] The local acidosis which develops in an inflamed area was stressed particularly by Schade[46] The inability of polymorphonuclear leukocytes to survive when the hydrogen ion concentration is high was shown by the writer to occur both *in vivo* and *in vitro*[29, 47] The local acidosis was found referable to a glycolytic process so that a true lactic acidosis develops with the progress of the acute inflammation[30] In brief the pH conditions the cytological sequence which in turn is influenced by changes in the intermediary carbohydrate metabolism The lymphocytes appear to be unaffected by the developing local acidosis When the pH reaches a level below 6 0 or 5 5 many of the leukocytes including the macrophages are to a large extent incapable of survival and frank pus results Pus formation therefore in acute inflammation seems to be a function of the hydrogen ion concentration In a cold abscess as in tuberculosis or in a long standing inflammation the pH of the exudate tends to be alkaline in nature[30] In conclusion it is important to keep in mind that the acute inflammatory process is a dynamic phenomenon which at its initial stage is locally at an alkaline pH to be subsequently followed by an acid pH As we shall see some significant changes occur in the inflamed area with this progressive development

Harris in a recent review[14] casually dismisses the relation of the pH to the cytology in an acute inflammation in a somewhat unwarranted manner as follows On the present evidence it seems fair to say that no connection between the pH and the cytology of inflammatory exudates has been established This author in his discussion entirely ignores the confirmatory works of Rugiero and Tanturi[48] and of Bennett[49] even though these investigators are cited in the writer s monograph[16] to which Harris refers in his review Furthermore Harris takes no cognizance of all the additional studies of the writer on carbohydrate metabolism[30] in which it was definitely shown that the pH changes are in turn referable to a disturbance in glycolysis and that with the development of a true lactic acid acidosis the polymorphonuclears are incapable of survival This author also fails to consider the *in vitro* studies with various pH buffers *in which the writer showed the direct noxious effects on leukocytes of an acid* pH[7] Menkin had also pointed out that in the rabbit infected with tubercle bacilli the elevated lactic acid content in the tubercle reasonably accounts for the prevalence of mononuclear cells (Menkin[4] p 85) In regard to Harris s

[illegible]



FIGURE 6 Eff t th pot cy f le k ta d g d ped ft tw d ly j t f C m pou d F (hydroc t so e) to th infl m d pl l ty f d g A t tal f 40 mgm f the t l w j ct d F ll wi g th two j t f the t t d th t t d l k ta w capabl of ind g in a rabbit any diaped s (/ eff t w th th t in tr l a h w in FIGURE 3) X410

flamed tissue migrate to a large extent from the blood[55] Recently Cooke Golding and Kahn seem to have thrown further light on this controversial point[56] These investigators first injured the skin of a rabbit and then killed the animal and incubated the excised skin These samples of tissue were removed from the body and in spite of having no control from the central nervous system and no circulation they displayed perivascular infiltration The changes resembled those of a chronic inflammation These changes developed within a few hours It appears that the perivascular infiltration arises from *in situ* changes of adventitial macrophages

3 The Factors Which Determine the Leukocyte Level in the Circulation with a Concomitant Acute Inflammation

I should like now to discuss as briefly as possible the effect of an acute inflammatory reaction on the systemic white blood cell level When an acute inflammation is induced in the pleural cavity of a dog with a chemical irritant it is difficult to predict as a consequence the outcome on the number of leukocytes in the circulation[57] Some data bearing on this question have been assembled in TABLE 1 It is clear that an inflammation of one to four days duration and with its exudate at an alkaline neutral or acid pH reflects in the blood stream either a leukocytosis a leukopenia or no appreciable change in the white blood cell count Let us examine the possible mechanisms involved in this unpredictable hematological picture

(1) What are the factors which induce a leukocytosis concomitant with an acute inflammation? There are at least two factors to be discussed

(a) When an acute pleural inflammation develops a leukocytosis the whole or cell free exudate from such an inflamed area injected in turn intravascularly into a normal dog will tend to induce a state of leukocytosis[58] Neither leukotaxine nor blood serum is capable of producing any such effect There is evidently present in inflammatory exudates (particularly exudates at an alkaline pH) a leukocytosis promoting factor (abbreviated as LPF)[58] Without going too much into the details which have already been published[59 18 59 60] this

TABLE 1
THE PRESENCE OF AN EXUDATE AT VARYING HYDROGEN ION CONCENTRATIONS AND THE NUMBER OF CIRCULATING LEUKOCYTES

D g N	App m t d t f fl mm t day	B sal wh t bl od ll t bef d g fl mm t pe cu mm	pH f d t	Wh t blood ll t w th fl mm t t d d t pH pe mm
110-T	1	9 350	7 0	21 400
120-T	1	12 850	7 3	21 900
121 T	3	27 050	7 5	26 200
132 T	3	14 500	7 6	27 600
121 T	4	27 050	6 95	10 500
110-T	2	9 350	6 5	32 250
87 T	4	11 100	6 7	9 050
130-T	4	7 800	6 87	55 150

k pe t t l l l d ta t ll th t p t w t l zed (d t t k m tw table n M k V 1949 Blood J H m t l 4 (1323)

comments concerning the studies of Steinberg and Dietz[50] it should be remembered that these investigators used a wholly different method from the one employed by the writer These workers failed to correlate the cytology and the pH on *identical* samples of exudative material This fact had been pointed out by Menkin in a critical survey of the study (Menkin[47] footnote pp 116 and 117) This reply is not even considered by Harris Finally, in regard to Lurie's work no mention is made by Harris of the following comments and observations made by Menkin and Warner in 1937[30] The observations reported here and in a preceding communication have all been made on dogs They have consistently revealed the fact that the pH seems to condition the cytological picture in inflammation However in some unpublished observations on the exudates of rabbits the correlation obtained between the cytological picture and the pH of the exudate was not as consistent as observed in dogs Similar findings have recently been noted by Lurie[51] It is interesting in this connection that, in a review of intracellular digestion Opie[5] cites the studies of various investigators who have either failed or found considerable difficulty in demonstrating proteolytic enzymes (readily demonstrable in dogs) in the leukocytes of rabbits' (Menkin and Warner[30] footnote p 28) Lurie, at first was able to observe the same cytological sequence correlated with the pH in guinea pigs that the writer had found in dogs[53] In a later study on rabbits and guinea pigs Lurie could not demonstrate a constant correlation, although the guinea pig seemed to show a slightly better correlation[54] It is quite evident from the observations of Lurie and of Menkin and Warner[30] that the rabbit leukocytes do not respond to pH changes in an acutely inflamed area as they do in the dog The possible reduction or absence of certain enzymes in the rabbit leukocytes may perhaps account for the difference in behavior Nevertheless as pointed out above it seems that in the rabbit the changes in intermediary carbohydrate metabolism at the site of inflammation with the corresponding production of lactic acid may perhaps be adequately correlated with the cytological picture As mentioned this seems to be the case in tuberculous lesions of rabbits (Menkin[4] p 85) Finally Harris has misunderstood the studies of the writer[9, 47, 30] by stating that Menkin modified his position' (Harris[14] p 553) by inferring that the writer had ever conceived that the pH in an acutely inflamed area had to do with anything but a correlation of the actual local cytological picture The cellular constituents in the inflamed area had originally been described in 1934 as is often done in pathology *i e* as an infiltration[9] but this description never referred to the actual mobilization of cells into the area of inflammation In order to obviate this possible misinterpretation the writer has subsequently and categorically stated that it is a question of survival of leukocytes at different pH levels and not one of mobilization (see footnote p 25 Menkin[16]) As stated above however it appears that leukotaxine may perhaps also be concerned in the mobilization of mononuclear phagocytes at the site of inflammation

) Do mononuclear cells in inflammation arise *in situ* as many have believed? The writer has expressed the view that in his experience mononuclear cells are seen to migrate but rather infrequently through the capillary wall[4] Maximow and Bloom on the other hand believe that the mononuclear cells in in

TABLE 2

EFFECT OF LEUKOCYTOSIS PROMOTING FACTOR (LPF) FROM DIFFERENT TISSUES ON THE NUMBER OF CIRCULATING WHITE CELLS

Dog	T su	Dose mgm	P t se WBC f j t f LPF cu mm
273 T	Liver	13 cc	57 7
246-T		5 cc	134 3
		5 cc	1 0 8†
319 T		5 cc	149 0
325-T		9 cc	20 4
326-T		50 mgm	21 3
309 T		75-85 mgm	27 3
171 T	Spleen	25 cc	58 4
249 T		5 cc	63 2
317 T		29 cc	82 4
325 T		30 cc	57 5
326-T		60 mgm	50 0
244-T		6 cc	42 1
234-T		8 cc	47 5
309 T	Kidney	10 cc	76 6
320-T		18 5 cc	71 6
303 T		18 5 cc	56 [illegible]
329 T		3 5 cc	67 8
		17 cc	58 2
309 T		50 mgm	10 0
		8 cc	36 3
313 T	Muscle	10 cc	72 2
		17 cc	37 8
314-T		12 cc	164 3
320-T		8 5 cc	96 0
314-T		4 cc	52 3

Ly ph l zed m t l
† Le l f WBC 29 h ft dm t t f LPF

Finally observations were also made on the production of an active LPF following repeated injections into an area of acute pleural inflammation of hydrocortisone (Compound F 10 to 20 mgm per injection) Here again as in the case of leukotaxine the activity of the injured cell appears to be appreciably suppressed [73, 4-a, 4 b] The LPF recovered from the corticosteroid treated inflamed area is about half as active as that recovered from the control inflamed pleural cavity (TABLE 3 FIGURE 7) The data indicate also an effect on the bone marrow for the discharge of immature leukocytes into the circulation after repeated injections of Compound F into the inflamed area is definitely reduced (TABLE 4)

Compound F does not interact directly with the LPF and thus suppress the activity of this substance Three experiments were made in which hydrocortisone in concentrations ranging from 15 to 21 mgm was mixed *in vitro* and allowed to stand for 18 to 19 minutes with the leukocytosis promoting factor The LPF treated in this way induced an average increase of 130 1 per cent in the number of circulating leukocytes in comparison with an average rise of 111 4 per cent in two control dog experiments injected with the LPF alone

factor is thermolabile, and electrophoretic studies have revealed it to be distributed between the alpha$_1$ and alpha$_2$ globulins[61] Subsequent studies on aged and apparently inactive LPF have demonstrated that spontaneous denaturation occurring probably by aging the material, has split the active principle from the rest of the molecule[67] The leukocytosis promoting factor has now become not only an inactive substance, but also a very insoluble substance Suspending it in an aqueous medium and centrifuging reveals that the original potency can be regained but only in the supernatant phase This supernatant fraction is thermostable and appears to be a polypeptide, judging from studies on the amino nitrogen before and after hydrolysis and from unpublished chromatographic investigations recently undertaken by my associate Doctor W Kalnins * Our present view of the leukocytosis promoting factor (LPF) is of a thermolabile alpha globulin which upon denaturation induced by standing several months in a dessicator dissociates As a consequence the LPF liberates the active principle in the form of a heat stable polypeptide I shall return presently to the remaining part of the molecule following its dissociation by apparent denaturation The LPF offers various possibilities of clinical application such as perhaps reinforcing antibiotics[63] for it was pointed out a number of years ago that the prognosis of many inflammatory processes depends on the number of circulating leukocytes[64] Recently Storer Sanders and Lushbaugh tried the LPF in X ray irradiated mice[65] They found little significant protection even though the number of circulating leukocytes was definitely increased after administration of the LPF Scrutiny of their data reveals some protection with the lower doses of X ray utilized (450 r) It is however to be recalled that survival like resistance is probably the resultant of several factors It may well be that X ray irradiation not only affects the hematopoietic tissue but that also there may be released some necrosin[66, 67] besides some other unknown factors (*e g* splenic factor of Jacobsen)[68]

Canine LPF derived from inflammatory exudates is also active in human beings[69, 70] Recently we have also shown that a saline extract of severely injured dog liver spleen kidney or muscle tissue yields upon extraction by similar methods as utilized for the LPF of exudates[10] a potent fraction of LPF (TABLE 2) Saline *per se* is ineffective on the number of circulating leukocytes whereas the LPF of these tissue extracts appreciably enhances the white blood cell level Precisely as in the case of leukotaxine[13] cellular injury seems to be the only requisite for the formation of an active LPF Lyophilization tends to inactivate this tissue LPF except perhaps in the case of the splenic LPF (TABLE 2) This finding is in contrast to the findings with the LPF of inflammatory exudates

Another important attribute of the LPF from the exudates is its hyperplastic effect on the bone marrow[71] Relatively few to about 100 mgm of the material will cause not only a discharge of immature polymorphonuclear leukocytes into the blood stream[16, 7] but also within two days or so a marked growth effect in the marrow specifically affecting the granulocytes and to some extent the megakaryocytes[71]

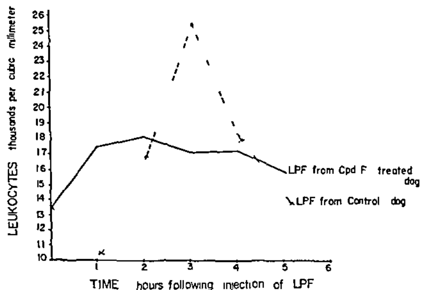
* P pe part t [illegible] hrom togr phy re [illegible] t12 d [illegible] t m [illegible] prese t th [illegible] kocytos p m t g m t [illegible] Th se m [illegible] are ly [illegible] p [illegible] m th [illegible] ty os thr n gly [illegible] se [illegible] gl tam acid d part [illegible] d

The thermostable factor on the other hand is associated with the euglobulin fraction of acid exudates in the later stages of inflammation[67] It is closely bound to pyrexin the pyrogenic factor of exudates This thermostable leukocytosis factor can be dissociated from pyrexin and can actually be crystallized[75] For its dissociation from pyrexin and its crystallization see SCHEME 3

The crystals appear in the gross as needlelike spicules and microscopically they *present themselves as irregularly shaped doubly refractile crystals* The crystals induce a rise of over 40 per cent in the number of circulating leukocytes[75] The leukocytosis is in part referable to a discharge into the circulation of immature or 1 lobe form of granulocytes[75] The effect of the factor on the bone marrow is at present under study Preliminary study indicates that there is some degree of hyperplasia and mitotic activity in the marrow In contrast to the thermolabile LPF, the crystals of the thermostable factor induce no change in the amino nitrogen when compared before and after hydrolysis[75] This fact would suggest that the material is not polypeptide in character The total nitrogen of the crystals is 2 1 per cent in comparison with a content of 12 3 per cent nitrogen in the thermolabile LPF

In brief the two described factors promoting leukocytosis *i e* the thermolabile LPF of alkaline exudates and the thermostable leukocytosis factor of acid exudates reasonably explain the developing systemic leukocytosis throughout the duration of the acute inflammatory reaction from its initial alkalinity to its acid stage

(2) What factors are involved in the development of *leukopenia* with a concomitant acute inflammation? At least two factors are involved



F [illegible] (LPF) [illegible] Compound F [illegible] The [illegible] of LPF [illegible] the Compound F [illegible] The [illegible] Goldman [illegible]

TABLE 3

EFFECT OF LEUKOCYTOSIS PROMOTING FACTOR (LPF) OF EXUDATES FOLLOWING INJECTION OF COMPOUND F (FREE ALCOHOL) INTO AN INFLAMED AREA‡

Exp No	Dog No of LPF	Amt of LPF inj cc	Total amt Cpd F admin mgm	Basal white blood cells/mm	Highest white blood cell count mm	% increase in white blood cells
1 E	256-T	30	41 9	10500	198 0	89 0
C		30	—	7225	18650	158 1
2 E	8 D	20	20	8225	12450	51 4
C		±18	—	8700	15450	77 6
3 E	171 T	20	20	14125	18450	30 6
C		20	—	11900	19300	62 2
4 E†	249 T	21	40	13325	20750	55 7
5 E†	264 T	20	40	7500	11100	48
6 E	267 T	25	24	11875	15650	31 8
C		±23	—	15825	28200	78 2
7 E	N	30	34 2	9550	143 0	50 3
8 E	282 T	24	11	13475	18100	34 3
C		±23	—	13500	25400	88 1
Avg E						48 9
C						92 8

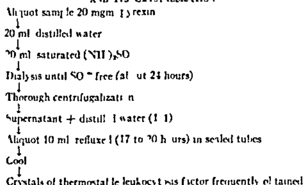
E = p r m t l C = C t l
† C mm al hyd o o t t l d th p n t in all th pe m t C mp d F (f alc h l) mpl y d
‡ Th fl mm t ry ct d d by th t pl l j t f 1 to 2 of t p t

This evidence supports further the view that hydrocortisone seems to reduce the effectiveness of the leukocytosis promoting factor when repeatedly injected directly into an inflamed area by suppressing the activity of the injured cell rather than by interacting with any preformed LPF at the site of inflammation

This distinction has not been as clearly demonstrated with leukotaxine Cortisone had also been shown to act directly on leukotaxine when the steroid was either mixed *in vitro* or injected intravenously [74] Under such circumstances it was shown that cortisone after repeated injections penetrates into an inflamed focus and once there seems to repress leukotaxine activity [74] It is conceivable that cortisone suppresses both leukotaxine *per se* and its formation by injured cells at the site of inflammation Nevertheless in view of the cellular suppressing activity of Compound F on *Arbacia* ova described above [41] and the inability of this corticosteroid to suppress the LPF when mixed *in vitro* it is the belief of the writer that the primary anti inflammatory mechanism is at a cellular level and is referable to a suppression of the activity of injured cells

(b) Another factor which promotes leukocytosis present in acid inflammatory exudates is the thermostable leukocytosis factor [57, 75] It will be recalled that the LPF was primarily found in alkaline exudates at the beginning of the inflammatory reaction and that it was associated with the alpha globulins

SCHEME 3 FOR DISSOCIATION OF THE THERMOSTABLE LEUKOCYTOSIS FACTOR FROM PYREXIN AND ITS CRYSTALLIZATION



companying many inflammatory processes is caused by a trapping mechanism referable in turn to a cumulative action of the leukopenic factor that is liberated from the site of inflammation

Wintrobe and his collaborators have essentially confirmed the presence of the leukopenic factor in an inflamed area[79] These investigators have used a different method of extraction from that originally described by the writer Their studies were made on the rat They utilized a saline extract of an abscess as the source of their leukopenic factor Wintrobe and his associates have also reported a depression of both circulating polymorphonuclear leukocytes and mononuclears Their factor is also thermostable and nondialyzable There is but little doubt that the leukopenic factor described by the writer is similar to the one reported by Wintrobe and his collaborators[9]

Steinberg and Martin by the injection of milk into rabbits report that the initial leukopenia is referable to vascular stasis and the adhesion of granulocytes to the vascular endothelium[80] Subsequently there is an actual trapping or as these authors term it a sequestration of leukocytes in the spleen liver and lung By observing granulocytic disintegration in tissues and blood these authors arrive at the concept pointed out and first proved by the writer that cellular injury as displayed in inflammation liberates various chemical factors some of which are significant in explaining leukopenia when it is one of the biological manifestations of inflammation[77, 81, 16]

In brief we have described four factors involved in regulating the white blood cell level accompanying an acute inflammation Two of these factors tend to elevate the number of circulating white cells to wit the thermolabile leukocytosis promoting factor and the thermostable leukocytosis factor Two other factors have an opposite effect They tend to depress the level of circulating leukocytes They are the thermolabile leukopenin of alkaline exudates and the thermostable leukopenic factor of acid exudates The more exact chemical identification and properties of these four factors await further studies The significant concept which I should like to leave with you however is the following we have seen that the white blood cell level in a dog accompanying

TABLE 4

EFFECT OF COMPOUND F (FREE ALCOHOL) INJECTIONS INTO INFLAMED AREA ON DISCHARGE OF IMMATURE POLYMORPHONUCLEAR LEUKOCYTES (1 LOBE FORM) IN CIRCULATING BLOOD FOLLOWING INJECTION OF THE LEUKOCYTOSIS PROMOTING FACTOR (LPF) RECOVERED IN TURN FROM EXUDATIVE MATERIAL‡

Exp No	Experimental			Control		
	Amount of Compound F injected into inflamed pleural cavity mgm	Basal No of 1 lobe form in blood stream %	Highest No of 1 lobe form in circulation following injection of either 15 cc or about 20 cc of LPF %	Amount of saline injected into inflamed pleural cavity cc	Basal No of 1 lobe form in blood stream %	Highest No of 1 lobe form in circulation following injection of either 15 cc or about 20 cc of LPF %
1	20	18	34	8	10	40
2	24	34	40	4	38	70
3	22 2	24	34	6 4	24	52
4	11	10	28	3	8	58
5†	20	24	32	8	12	52
Avg	19 4 mgm	22%	33 6%	5 9 cc	18 4%	54 4%

* About 5 cc of whole exudate injected into circulation of test dog instead of extracted LPF from exudative material

† LPF extracted in this experiment from 1 cc of exudate; in the other experiments the LPF was recovered from about 20 cc of exudative material

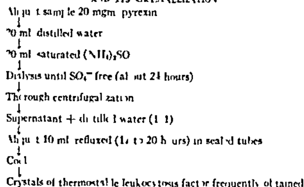
‡ Inflammation induced by 1 to 2 cc of turpentine into the right pleural cavity

(a) The injection of an alkaline exudate is frequently accompanied by an initial decrease in the total number of circulating white cells This decrease seems to be referable to a thermolabile factor [6] Boiling the exudate obliterates to some extent the initial drop in the white blood cell level As previously described the thermolabile LPF upon aging denatures and dissociates into an active supernatant fraction and a relatively insoluble precipitate [6] This latter part of the denatured complex is thermolabile and has the property of decreasing the number of circulating leukocytes This factor has been termed *leukopenin* and is probably the same factor encountered in alkaline exudates [76] Leukopenin is associated with the globulin fraction of alkaline exudates

(b) An acid exudate likewise induces an initial leukopenia when injected into the circulating blood of a dog [77] This effect can also be duplicated by crude pyrexin the pyrogenic factor recovered in the euglobulin fraction of acid exudates [77] Boiling fails to inactivate the effect indicating that one is dealing in acid exudates with a factor different from the leukopenin of alkaline exudate

This leukopenic factor can be dissociated from crude pyrexin by partial hydrolysis of the latter with 0 1 N HCl for about 15 minutes adjusting to about pH 10 with N NaOH concentrating and dialyzing off the electrolytes and other diffusible material Upon evaporation to dryness or lyophilization an active leukopenic factor is obtained which has only leukopenic properties with essentially no pyrogenic capacity [7] The mechanism of leukopenia induced by the leukopenic factor appears to be referable to a trapping of granulocytes and mononuclears in the alveolar walls of the lung the sinusoids of the liver and in the spleen [78] It is conceivable that the acute splenic tumor ac

SCHEME 3 FOR DISSOCIATION OF THE THERMOSTABLE LEUKOCYTOSIS FACTOR FROM PYREXIN AND ITS CRYSTALLIZATION



companying many inflammatory processes is caused by a trapping mechanism referable in turn to a cumulative action of the leukopenic factor that is liberated from the site of inflammation

Wintrobe and his collaborators have essentially confirmed the presence of the leukopenic factor in an inflamed area [79] These investigators have used a different method of extraction from that originally described by the writer Their studies were made on the rat They utilized a saline extract of an abscess as the source of their leukopenic factor Wintrobe and his associates have also reported a depression of both circulating polymorphonuclear leukocytes and mononuclears Their factor is also thermostable and nondialyzable There is but little doubt that the leukopenic factor described by the writer is similar to the one reported by Wintrobe and his collaborators [79]

Steinberg and Martin by the injection of milk into rabbits report that the initial leukopenia is referable to vascular stasis and the adhesion of granulocytes to the vascular endothelium [80] Subsequently there is an actual trapping or as these authors term it a sequestration of leukocytes in the spleen liver and lung By observing granulocytic disintegration in tissues and blood these authors arrive at the concept pointed out and first proved by the writer that cellular injury as displayed in inflammation liberates various chemical factors some of which are significant in explaining leukopenia when it is one of the biological manifestations of inflammation [77, 81, 14]

In brief we have described four factors involved in regulating the white blood cell level accompanying an acute inflammation Two of these factors tend to elevate the number of circulating white cells to wit the thermolabile leukocytosis-promoting factor and the thermostable leukocytosis factor Two other factors have an opposite effect They tend to depress the level of circulating leukocytes They are the thermolabile leukopenin of alkaline exudates and the thermostable leukopenic factor of acid exudates The more exact chemical identification and properties of these four factors await further studies The significant concept which I should like to leave with you however is the following we have seen that the white blood cell level in a dog accompanying

an acute pleural inflammation caused by a chemical irritant varies (TABLE 1), and that the final level is quite unpredictable It seems that the final white blood cell level is a resultant of the four aforementioned factors The predominance of the leukocytosis factors is accompanied by a leukocytosis On the contrary a preeminence in the concentration of the leukopenic factors is followed by a leukopenia [57] A balance of these opposing tendencies results in no appreciable change in the number of circulating leukocytes with a concomitant acute inflammation *

Finally before terminating this survey I should like to point out that as already described an acute inflammatory reaction proceeds from an alkalinity to an acidity Despite these local changes in reaction a state of systemic homeostasis tends to be maintained throughout the duration of an acute inflammation At the alkaline stage the leukocytosis is referable to the *leukocytosis promoting factor* (*LPF*) whereas in the acid stage of inflammation the leukocytosis is determined by the *thermostable leukocytosis factor* Leukopenia with inflammation is referable in the initial alkaline stage to the thermolabile *leukopenin* whereas in the acid stage the systemic leukopenia is ascribed to the liberation by the injured cells of a *leukopenic factor* It is by these delicate regulating chemical mechanisms at the dynamic site of an acute inflammation that a systemic homeostasis in the organism as a whole is maintained throughout the duration of inflammation [3, 8]

Summary and Conclusions

This presentation involves a consideration of several distinct problems

(1) The present status of leukotaxine and the significance of this substance in explaining the migration of polymorphonuclear leukocytes and possibly the mobilization of mononuclear phagocytes in an acute inflammation are considered Observations based on recent chromatographic studies reaffirm the view that leukotaxine appears to be a polypeptide Enzymatic studies on the digest of proteins point to the formation of intermediary protein breakdown products which are peptides These peptides induce increased capillary permeability and diapedesis of leukocytes These two properties have been dissociated by Spector in the digest of fibrin Dissociation of these two properties of leukotaxine however have failed to be effected in the peptides of inflammatory exudates Consequently leukotaxine is considered at present at least to be a single entity New and convenient methods of isolating leukotaxine are described

Moo d T h k tly rep t d th t t j th bb t h p th umb r of l t g l k yt Aq t t f t d d mod t to l k yt Th y f d th t th w ll p d d by rv b f d f l k j N tt mpt w m l b th se k t l t th tw f t th t l k yto (LPF d th th m t bl f t) d th tw f t th t d le k p (l k p l th l k pe f t) Th f t th t p od t f r t d g t t d d t p d e m la p t t f ll p g A h b p t d t b d p ly th p i t m y p h p t som se ly th h m l f t d f m dat Th f h w t t ll se t l f t m pl bl t g th r t p d t f d g t t g fl mm t y ta t wh h t l b t th h m l f t f m th w j d ll Th re ry f th f t f m th q t t f t w ld pr t th ew j t p se l by th w t M k h f und th t b th by l g b l k t l k m t l f m th l t t f m l t I p l g se t f th p se t m pt m l b t j t d w th th LPF d f m t t t (2) Th ry g lt f M d T h k m y w ll b f bl t th d ff t pe t t t th t t f th l kocyt d l k pe f t d bed b

(2) The significance of the developing acidosis in an acute inflam nation in conditioning the cytological picture is considered The changes in the hydrogen ion concentration of the exudate are in turn referable to a disturbance in the local intermediary carbohydrate metabolism The mechanism of pus formation in an acute inflammation is pointed out The pH mechanism involves only survival of leukocytes in the sequence of polymorphonuclear cells as they are replaced by macrophages in the progress of the developing inflammatory reaction

(3) The unpredictable picture of the white blood cell level with a concomitant acute inflammation is pointed out The significance of various local factors liberated by the injured cell at the site of inflammation is considered It is shown that leukocytosis is referable to at least two factors (a) the thermolabile leukocytosis-promoting factor (LPF) of alkaline exudate and (b) the thermostable leukocytosis factor of acid exudate Leukopenia is shown to be due also to at least two factors (a) leukopenin found in an alkaline exudate and (b) the leukopenic factor of acid exudate The ultimate hematological picture seems to be a resultant of these four factors A predominance of the leukocytosis factors results in an elevation in the number of circulating leukocytes A pre-eminence of the leukopenic factors induces a depression in the level of white blood cells while a balancing effect of all the factors may produce no appreciable change

(4) The developing acidosis in a progressive acute inflammation with the corresponding production of different chemical factors but with similar biological properties at varying local hydrogen ion concentration induces no change in homeostasis as far as the organism as a whole is concerned

(5) The anti inflammatory mechanism by hydrocortisone (Compound F) is considered Leukotaxine and the leukocytosis-promoting factor are shown to be either inactivated or markedly reduced in potency following repeated injections of this corticosteroid into the inflamed area Earlier observations on *Arbacia* ova have indicated that the anti inflammatory steroids suppress cell activity These and the present observations as well as the inability of hydrocortisone to affect the potency of the LPF when mixed *in vitro* lead to the view that the primary anti inflammatory mechanism is at a cellular level By suppressing cellular activity the chemical factors liberated by the injured cell which *per se* are responsible for the actual manifestation of inflammation seem to be repressed in their formation

Bibliography

1 Menkin V 1936 J Exptl Med 64 485
2 Menkin V 1938 J Exptl Med 67 129
3 Menkin V 1938 J Exptl Med 67 145
4 Menkin V 1940 Dynamics of Inflammation (Reprinted 1950) Macmillan New York N Y
5 Morimoto M 1953 Naika No Ryoiki 1(5) 401
6 Menkin V 1944 Alexander s Colloid Chemistry Theoretical and Applied 5 917 Reinhold New York N Y
7 Menkin V 1938 J Exptl Med 67 153
8 Duthie E S & E Chain 1939 Brit J Exptl Path 20 417
9 Cullumbine H & H N Rydon 1946 Brit J Exptl Path 27 33

an acute pleural inflammation caused by a chemical irritant varies (TABLE 1), and that the final level is quite unpredictable. It seems that the final white blood cell level *is a resultant of the four aforementioned factors*. The predominance of the leukocytosis factors is accompanied by a leukocytosis. On the contrary, a preeminence in the concentration of the leukopenic factors is followed by a leukopenia.[57] A balance of these opposing tendencies results in no appreciable change in the number of circulating leukocytes with a concomitant acute inflammation.*

Finally, before terminating this survey, I should like to point out that, as already described, an acute inflammatory reaction proceeds from an alkalinity to an acidity. Despite these local changes in reaction, a state of systemic homeostasis tends to be maintained throughout the duration of an acute inflammation. At the alkaline stage, the leukocytosis is referable to the *leukocytosis promoting factor* (*LPF*), whereas in the acid stage of inflammation the leukocytosis is determined by the *thermostable leukocytosis factor*. Leukopenia with inflammation is referable, in the initial alkaline stage, to the thermolabile *leukopenin*, whereas in the acid stage the systemic leukopenia is ascribed to the liberation by the injured cells of a *leukopenic factor*. It is by these delicate regulating chemical mechanisms at the dynamic site of an acute inflammation that a systemic homeostasis in the organism as a whole is maintained throughout the duration of inflammation.[23, 8]

Summary and Conclusions

This presentation involves a consideration of several distinct problems.

(1) The present status of leukotaxine and the significance of this substance in explaining the migration of polymorphonuclear leukocytes and possibly the mobilization of mononuclear phagocytes in an acute inflammation are considered. Observations based on recent chromatographic studies reaffirm the view that leukotaxine appears to be a polypeptide. Enzymatic studies on the digest of proteins point to the formation of intermediary protein breakdown products which are peptides. These peptides induce increased capillary permeability and diapedesis of leukocytes. These two properties have been dissociated by Spector in the digest of fibrin. Dissociation of these two properties of leukotaxine, however, have failed to be effected in the peptides of inflammatory exudates. Consequently, leukotaxine is considered, at present at least, to be a single entity. New and convenient methods of isolating leukotaxine are described.

[illegible]

64 ROBERTSON D H & J P FOX 1939 J Exptl Med 69 229
65 STORER J B P SAUNDERS & C C LUSHBAUGH 1952 Proc Soc Exptl Biol Med 81 541
66 MENKIN V 1943 Arch Path 36 269
67 MENKIN V 1950 Proc Soc Exptl Biol Med 75 350
68 JACOBSEN L O E L SIMMONS L K MARKS L O GASTON M J ROBSON & J H ELDRIDGE 1951 J Lab Clin Med 37 683
69 MENKIN V 1946 Arch Path 41 376
70 MENKIN V E ULLED & F G GOODMAN 1946 Federation Proc 5 226
71 MENKIN V 1943 Am J Path 19 1021
72 MENKIN V M A KADISH & S C SOMMERS 1942 Arch Path 33 188
73 MENKIN V 1954 Science 120 1026
74 MENKIN V 1953 Brit J Exptl Path 34 412
75 MENKIN V 1950 Proc Soc Exptl Biol Med 75 378
76 MENKIN V 1948 Arch Path 46 145
77 MENKIN V 1946 Arch Path 41 50
78 MENKIN V 1946 Arch Path 42 154
79 KEMP I G E CARTWRIGHT & M M WINTROBE 1951 Am J Physiol 165 534
80 STEINBERG B & R A MARTIN 1954 Arch Path 57 227
81 MENKIN V 1947 Science 105 538
82 MENKIN V 1952 J Suisse Med 82nd Year 46 1184

10 SPECTOR W G 1951 J Path Bact 63 93
11 MENKIN V 1953 Arch Exptl Path Pharmakol 219 473
12 HARRIS H 1953 J Path Bact 66 135
13 MENKIN V 1952 Proc Soc Exptl Biol Med 80 223
14 HARRIS H 1954 Physiol Revs 34 529
15 MENKIN V 1953 Intern Arch Allergy Applied Immunol 4 131
16 MENKIN V 1950 Newer Concepts of Inflammation Thomas Springfield Ill
17 MENKIN V 1949 Nature 163 327
18 PASQUALI L 1947 Ricerche e studi med sper 18 209
19 MENKIN V 1937 Arch Path 24 65
20 MENKIN V 1937 Am J Path 13 633
21 MENKIN V 1940 Am J Physiol 129 691
22 MENKIN V 1942 Proc Soc Exptl Biol Med 51 39
23 MENKIN V 1951 Am J Physiol 166 509 518
24 MENKIN V 1940 Proc Soc Exptl Biol Med 44 588
25 PFOEHL J 1898 Zentr Bakt Parasitenk 24 343
26 RUCHLADEW N 1910 Z Biol 54 533
27 McCUTCHEON M 1942 Arch Path 34 167
28 FLECK L & D Borecha 1946 Ann Universit Mariae Curie Sklowdoski 1 335
29 MENKIN V 1934 Am J Path 10 193
30 MENKIN V & C P WARNER 1937 Am J Path 13 25
30a MENKIN V 1938 Physiol Revs 18 366
31 TERSHAKOVEC G A & V H MOON 1952 Am J Path 28 527
32 MOON V H & G A TERSHAKOVEC 1954 Arch Path 58 285
33 MOON V H C G GRAND & G A TERSHAKOVEC 1954 Arch Path 57 44
34 HOENE P & ROCHA E SILVA 1954 Personal Communication to V W Adamkiewicz and included on p 277 of Dr Adamkiewicz s Ph D Thesis A Biochemical Contribution of the Study of Inflammation University of Montreal Montreal Canada
35 MILES A A & E M MILES 1952 J Physiol 118 228
36 FELDBERG W 1953 Proc Roy Soc Med 46 256
37 HALPERN B N & BENACERRAF B 1954 Allergy Ann Rev Med 5 167
38 BIOZZI G G MENÉ & Z OVARY 1951 Arch Intern Pharmacodynamie 86 335
39 PASHKINA T S 1952 Doklady Akad Nauk S S S R 86 609 (Taken from 1953 Excerpta Medica Sect V 6 698)
39a MOON V H & M A TERSHAKOVEC 1954 Proc Soc Exptl Biol Med 85 600
40 MENKIN V 1951 Am J Physiol 164 294
41 MENKIN V 1952 Biol Bull 103 305 1953 Proc Soc Exptl Biol Med 82 182
42a MENKIN V 1953 Federation Proc 12 Rev canad biol 12 239
42b MENKIN V 1953 The Mechanism of Inflammation 137 G Jasmin & A Robert Eds Acta Inc Montreal Canada
43 CLARK I 1953 J Biol Chem 200 69
44 WYMAN L C G I FULTON M H SHULMAN & L L SMITH 1954 Am J Physiol 176 335
45 BORREL A 1893 Ann inst Pasteur 7 593
46 SCHADE H P NEUKIRCH & A HALLERT 1921 Z ges exptl Med 24 11
47 MENKIN V 1939 Arch Path 27 115
48 RUGIERO H R & C A TANTURI 1942 Semana méd No 8 13
49 BENNETT I JR 1948 J Exptl Med 88 279
50 STEINBERG B & A DIETZ 1938 Arch Path 25 777
51 LURIE M B 1936 Arch Path 22 272
52 OPIE E L 1922 Physiol Revs 2 552
53 LURIE M B 1937 Am J Path 13 612
54 LURIE M B 1939 J Exptl Med 69 579
55 MAXIMOW A A & W BLOOM 1930 Textbook of Histology 144 Saunders Philadelphia Pa
56 COOKE J V D GOLDRING & L I KAHN 1953 J Exptl Med 97 651
57 MENKIN V 1949 Blood 4 1323
58 MENKIN V 1940 Am J Path 16 13
59 MENKIN V 1940 Arch Path 30 363
60 MENKIN V 1947 Proc 4th Intern Congress for Microbiol 322 (Rosenkilde and Bagger Copenhagen Denmark 1949)
61 DILLON M L G R COOPER & V MENKIN 1947 Proc Soc Exptl Biol Med 65 187
62 MENKIN V 1948 Blood 3 939
63 MENKIN V 1947 Arch Path 43 566

off as sedimentation proceeds is very rich in leukocytes and platelets This cell suspension is then added to the serum under study

This cell suspension must be carefully controlled under phase observation It is of the utmost importance that the cells remain separated samples displaying spontaneous agglutination of cells must be discarded Numerous factors such as centrifugation shaking and sedimentation favor the spontaneous agglutination of platelets and leukocytes and may lead to errors in evaluating the agglutinating power of serum The heparin solution also must be carefully controlled Some samples of this substance secured from various commercial sources have appeared capable of producing leukocyte agglutination

Technique Two drops of leukocyte suspension and two drops of the serum sample under investigation are mixed in hemolysis tubes Two sets of tubes are prepared one of which is kept at room temperature and the other in the 37 C oven Every 10 minutes a drop of the mixture is placed on a slide a coverslip is added and the preparation is examined under phase contrast for possible agglutination lysis phagocytosis *etc* With human subjects the serum donor s own cells were also examined

In addition in order to study the effect of complement or of some other thermolabile component heated sera were used as well in conjunction with cell suspensions washed with heated serum Fresh serum was added subsequently to such preparations

Cinephotomicrography Agglutination lysis and phagocytosis have been followed by time lapse cinematography at the rate of one frame every four seconds which at standard projection speeds corresponds to an acceleration of the processes studied by a factor of approximately 90 times Technical details of cinematography have been given elsewhere [1 4]

Some sera producing lysis too rapidly were diluted to a point where about 10 minutes were required for lysis to be achieved

II Results

(*I*) *Effects of antierythrocyte antibodies* We shall not expand here on cytological changes attending agglutination and lysis of the erythrocyte This development has been described elsewhere [5] We shall consider only phagocytosis [6]

This phenomenon is peculiar to only granulocytes and monocytes At first the leukocytes move about more or less actively among the red blood cells and push these bodies aside without sticking to them Then in some instances an erythrocyte is seen to adhere to the surface of a leukocyte at a very localized point The leukocyte continues to form new pseudopods which continue to push the erythrocyte aside The red blood cell which had become affixed to the leukocyte surface is pushed forward as the others are but because it is adhering to the white blood cell the adjacent half of the erythrocyte becomes intimately invested with a cuplike formation of hyaloplasmic sheets This hyaloplasmic cup advances and pushes the erythrocyte which being attached at one point on the leukocyte surface becomes stretched and assumes

CYTOLOGIC ASPECTS OF IMMUNOHEMATOLOGY A STUDY WITH PHASE CONTRAST CINEPHOTOMICROGRAPHY

By Marcel C Bessis
Centre National de Transfusion Sanguine Paris France

Phase contrast microscopy has led to progress of fundamental importance because it has made possible the study of the finest structures of living cells *Chromatin mitrochondria the centrosome and specific granulations* are clearly visualized by this method often more distinctly than in stained preparations

The value of phase contrast microscopy can be still further enhanced when this technique is used in conjunction with cinephotomicrography As a rule, cellular movements are extremely slow when compared to the type of motion we are accustomed to see on the human scale Indeed many cellular movements are so slow that even a trained observer cannot perceive them With cinephotomicrography it is possible to speed up motion by factors in the range of 30 to 6 000 times In many cases this procedure has allowed the observation of cell behavior modifications which had been unnoticed in the course of direct observation These studies however despite their fundamental nature have suffered from a lack of sharply visualized intracellular organelles These limitations have now been obviated by utilizing the phase microscope an instrument that has proved itself perfectly suited to the study of cellular dynamics With this instrument cinematography now allows a very precise analysis of all cellular and intracellular movements in various media or following various morphologic alterations of the smallest organelles [1]

This paper covers observations made using such techniques on the effects of antierythrocyte antileukocyte and antiplatelet sera derived from human or animal sources

I Materials and Methods

Source of sera Experimental sera were obtained from rabbit blood following immunization against erythrocytes leukocytes and platelets from normal or leukemic human blood Rabbits were injected every three days with 1 cc of washed cells suspension After six or eight injections a serum is obtained with an agglutinating titer often reaching 1000 for erythrocytes 128 for leukocytes and 64 for platelets These sera all contain a mixture of antileukocyte erythrocyte and platelet agglutinins Nevertheless they can be utilized *in vitro* without any further manipulations They can also be used and we have done this frequently after adsorption of the undesired agglutinins or following elution of pure specific antibodies (Bessis and Breton Gorius) [2]

Pathologic sera were obtained from patients with various dyscrasias such as Hodgkin s disease lymphoid leukemia [3] thrombocytopenic purpura lupus erythematosus *etc*

Separation of platelets and leukocytes Blood is drawn on heparin or citrate and is allowed to sediment of its own accord in inclined tubes Plasma pipetted

adhering to the leukocyte The phenomenon of bisecting and of engulfing may then take place with the red cell stroma

When a red blood cell or a portion of the cell has been engulfed one can see its intracytoplasmic hemolysis within a period of a few minutes to a few hours Hemoglobin diffuses suddenly out of the phagocytized cell into the digestive vacuole which enlarges steadily for about 10 minutes and then shrinks back and intimately surrounds the ingested cell

In summary phagocytosis of an erythrocyte or of its stroma appears to depend upon becoming adherent to a very localized point on the leukocyte surface This finding is in accordance with the well-established law of phagocytosis that the first prerequisite involves adherence of the phagocyte to the particle Without the sticking phenomenon the hyaloplasmic sheets push the erythrocytes about but do not succeed in enveloping them On the other hand whenever the cell sticks at one point the thrust of the leukocyte is adequate to stretch the erythrocyte into a pear shape under the conditions of our experiments

In the course of this stretching the peripheral area of the hyaloplasmic sheet (endowed of the same ability to contract as the rest of the cytoplasm) may bisect the erythrocyte The finding that hemolysis does not necessarily occur concurrently confirms previous findings of micromanipulation studies

Observations carried out to date lead us to believe this phenomenon is very generalized It might possibly explain the well known fact that ingested red cells are often of a smaller diameter than normal erythrocytes On the other hand one wonders whether schizocytes or at least some of them observed in certain cases of anemia might not be the result of this very process

(?) *Effects of antileukocytes antibodies* We shall describe here the main phenomena artificially dissociated Actually these phenomena take place concurrently

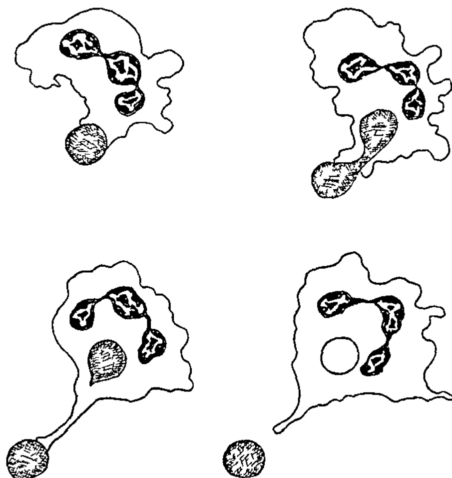
Agglutination Studies of agglutinated leukocytes under high magnification with the phase-contrast microscope reveal the following phenomena

When the cells are in good condition a situation that obtains when the experimental serum is not very active or when the medium has been heat deactivated (complement deactivation) and the proper spacing has been allowed between slide and coverslip a number of cells stick to the glass These cells can then be seen to form pseudopodia very actively and they tend to avoid each other Nevertheless they remain connected to each other through long filamentous structures similar to those found connecting agglutinated erythrocytes (Bessis and Bricka[6]) After about 10 minutes one can often observe a number of leukocytes in a rosette united to a central point by long radially distributed filaments (FIGURE 2) Such agglutinated cells often show signs of lysis and of phagocytosis These cells will be described below

Lysis When the antibody titer is very high and when the serum is relatively fresh (or when fresh serum is added) leukolysis can be demonstrated The antiserum effect can become evident within a few minutes of contact As a rule the following events take place the cell swells and seems to take up water Solation of the cytoplasm occurs as evidenced by the rapid Brownian movement of the cytoplasmic granules The nucleus at first retains a normal

a pear shaped appearance Then another process comes into play the hyaloplasm changes within the cuplike formation surrounding the elongated portion of the erythrocyte *Its appearance in phase contrast changes from light to* dark suggesting a transformation from sol to gel together with a local thickening of the hyaloplasmic sheet A dark ringlike structure forms at the edge of the hyaloplasmic cup This ring contracts rather rapidly, in a manner reminiscent of sphincter action This contracting ring cuts the erythrocyte into two portions the one enveloped by the hyaloplasmic cup becoming incorporated into the leukocyte cytoplasm and the other outside the sphincter it had evoked becoming free and resembling an erythrocyte of small diameter Occasionally this portion also sticks to the leukocyte surface (FIGURE 1)

This bisection of the erythrocyte by the leukocyte occurs usually without hemolysis Sometimes however the erythrocyte hemolyses shortly after



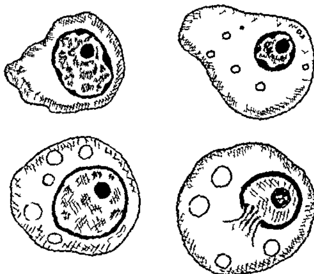
Fi u l T g (f m film t p seq) f th | tak d se t g f ryth ocyt by g a l yt I t cyt p la m hem ly t d th la t d gram

appearance but within a few minutes it begins to shrink and to undergo crinkling One gains the impression that the extensive swelling of the cytoplasm leads to compression of the nucleus A few minutes later however the nucleus also begins to swell the chromatin network becomes progressively fainter and there is a considerable increase in volume The nucleus is now spherical and appears completely bright and nucleoli, when present, are visualized as intensely dark structures

The cytoplasm continues to swell Specific granulations do not swell and their morphology appears unchanged They do display very active Brownian movement Mitochondria on the other hand are relatively nonmotile At first, they show warty swellings become distended collapse and often merge to form a large spherical vacuole Finally about 10 minutes from the beginning of the process the nuclear membrane yields usually at one point (sometimes at many points) The nucleoplasm then flows into the cytoplasm (FIGURE 3) The nucleoli flow through the small tear or tears in the nuclear membrane They swell and can then be seen to contain darker granules exactly comparable to those described in normal nuclei by various workers (Gonzales Guzman 1949 Estable *et al* 1951)

At the end of the process we have a completely amorphous mass in which a few granules still exhibit Brownian movement but in which none of the other cellular organelles can be identified

Phagocytosis Intact granulocytes monocytes and those hardly damaged by the antiserum phagocyti e the more severely damaged cells and occasionally, even phagocytize a cell which appears in very good condition As a rule,



F ur 3 Ly l luk cyt ty t serum Not tl lea kl g n th d g rat f m to h nd the sw ll g of tl cle a d fi lly th rupt f the l a memb n



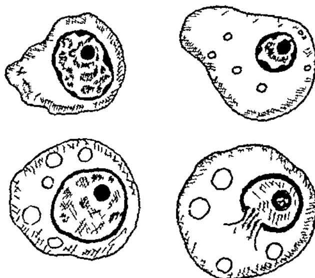
FIGURE 2 Le kocyt lump g f ll w g toa t serum t (f m A d (l) I th low ph t g ph a ell sh w m t d t g thre d se bet th ggl t t d l kocyt s (ph se co tra t)

appearance but within a few minutes it begins to shrink and to undergo crinkling One gains the impression that the extensive swelling of the cytoplasm leads to compression of the nucleus A few minutes later however the nucleus also begins to swell the chromatin network becomes progressively fainter and there is a considerable increase in volume The nucleus is now spherical and appears completely bright and nucleoli when present are visualized as intensely dark structures

The cytoplasm continues to swell Specific granulations do not swell and their morphology appears unchanged They do display very active Brownian movement Mitochondria on the other hand are relatively nonmotile At first they show warty swellings become distended collapse and often merge to form a large spherical vacuole Finally about 10 minutes from the beginning of the process, the nuclear membrane yields usually at one point (sometimes at many points) The nucleoplasm then flows into the cytoplasm (FIGURE 3) The nucleoli flow through the small tear or tears in the nuclear membrane They swell and can then be seen to contain darker granules exactly comparable to those described in normal nuclei by various workers (Gonzales-Guzman 1949 Estable *et al* 1951)

At the end of the process we have a completely amorphous mass in which a few granules still exhibit Brownian movement but in which none of the other cellular organelles can be identified

Phagocytosis Intact granulocytes monocytes and those hardly damaged by the antiserum phagocytize the more severely damaged cells and occasionally even phagocytize a cell which appears in very good condition As a rule



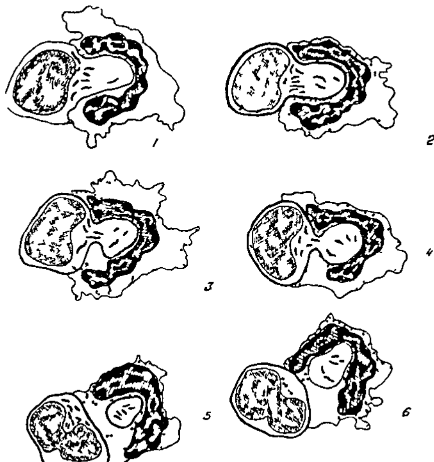
Fi ur 3 L s f leukocyt by t serum N t the le crinkl g in the dege rat o l m tochondria the sw g f the leu nd fi ally th rupture f th lear memb

however phagocytic cells focus on inactive cells and surround them more or less completely They often surround the damaged cell with a cup-shaped hyaloplasmic sheet deforming it into a pear shape, as if it were sucked into the center of this cup In other words the picture is identical to that described above for the phagocytosis of erythrocytes A portion of the cell is then cut off and digested by the phagocyte The other fragment is freed or may stick to a pseudopod for a time before being finally phagocytized as well (FIGURE 4)

The way in which granulocytes push and attempt to engulf another cell is very characteristic and strikingly visualized by cinemicrophotography (FIGURE 5)

Intact granulocytes may also phagocytize lysed cells, yielding cells identical to those described by Hargraves *et al* in lupus erythematosus[7]

Whenever the ingested cell or cell portion is in good condition, it retains its normal appearance for a while within the cytoplasm of the phagocyte Diges



Fi u 4 Sect f lymphocyt by g l cyt d g h gocytos Som m toch d se i th ge t d f gm t (f om film tr p seq ce)

tion begins after about 15 minutes Mitochondria degenerate the nucleus swells and the chromatin network becomes blurred The final state is identical to that which occurs following ingestion of lysed cells

A rare phenomenon but one which definitely occurs however consists of the ejection of a partially digested cell from a phagocyte The phagocyte then often gives evidence of being damaged or possibly even dying This









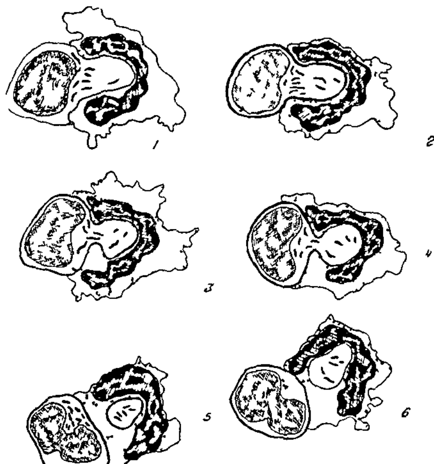
t F cu 5 D g am f g lxyt p h g l t lymph yt tt mpt g t s d t Th mo- y f qu t ph om (fr m hlm t p sequ ce)

however, phagocytic cells focus on inactive cells and surround them more or less completely They often surround the damaged cell with a cup shaped hyaloplasmic sheet deforming it into a pear shape as if it were sucked into the center of this cup In other words the picture is identical to that described above for the phagocytosis of erythrocytes A portion of the cell is then cut off and digested by the phagocyte The other fragment is freed or may stick to a pseudopod for a time before being finally phagocytized as well (FIGURE 4)

The way in which granulocytes push and attempt to engulf another cell is very characteristic and strikingly visualized by cinemicrophotography (FIGURE 5)

Intact granulocytes may also phagocytize lysed cells, yielding cells identical to those described by Hargraves *et al* in lupus erythematosus[7]

Whenever the ingested cell or cell portion is in good condition it retains its normal appearance for a while within the cytoplasm of the phagocyte Diges



F υ 4 Se t g of lymphocyt by g lxyt du g ph gocytos s S m m toch d se n th ing t d f gm t (f m a film t g seq)

Summary

The effect of erythrocyte leukocyte and platelet antisera has been described from phase contrast microscopy studies Special emphasis is placed on the sectioning of erythrocytes and leukocytes by phagocytes and on the dynamic aspects of agglutination and lysis Cytologic appearance has been found identical whether experimental or autoantisera are used

Acknowledgment

This manuscript submitted in French was translated into English by Doctor Jacques Padawer Department of Biology New York University

References

1 Bessis M 1954 Traité de cytologie sanguine vol 1 Masson et Cie Paris France
2 Bessis M & Breton Gorius J 1954 Flution des anticorps à partir des cellules osmiquées Compt rend soc biol 148 209–10
3 Andre R, B Dreyfus & M Bessis 1954 Anticorps anti leucocytaires dans un cas de leucém lymphoide associée à une mala li de Hodgkin Rev Hématol 9 50–67
4 Bessis M & M Bricka 1952 Aspect dynamique des cellules du sang Son étude par microcinématographie en contraste de phase Rev Hématol 7 407–35
5 Bessis M & M Bricka 1950 Etude au microscope électronique sur l hémolyse l agglutination la forme et la structure des globules rouges Rev Hématol 5 3–4 396–427
6 Policard A & M Bessis 1953 Fractionnement d hématies par les leucocytes au cours de leur phagocytose Compt rend soc biol 147 982–83
7 Hargraves M M H Richmond & R Morton 1948 Presentation of two bone marrow elements the tart cell and the L L cell Proc Staff Meetings Mayo Clinic 23 26–28
8 Wilson A T 1953 The ejection of phagocytized particles by leukocytes J Exptl Med 98 305–10

regurgitation of phagocytized material has recently been described by Wilson[5] during the phagocytosis of streptococci and of erythrocyte stroma

Effect of antiplatelet antibodies The appearance of platelets following contact with antiserum is identical to that just described for leukocytes At first the clumps are formed by normally motile cells but, after a while the platelets become lysed and characteristically appear as very clear small spheres, two to four micra in diameter within which float black granules These granules often are agglutinated (FIGURE 6) Granulocytes and monocytes then phagocytize these platelets in the manner described for leukocytes

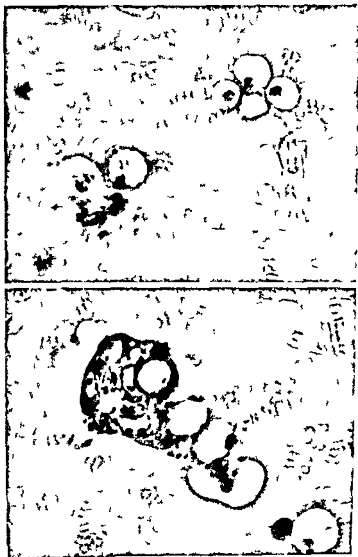


FIGURE 6 Lys[is] (top) a[n]d phagocytos[is] [(]bottom) [o]f pl[a]t[e]l[e]t[s] (f[r]om A[...] Ph[a]se [...])

Summary

The effect of erythrocyte, leukocyte and platelet antisera has been described from phase contrast microscopy studies Special emphasis is placed on the sectioning of erythrocytes and leukocytes by phagocytes and on the dynamic aspects of agglutination and lysis Cytologic appearance has been found identical whether experimental or autoantisera are used

Acknowledgment

This manuscript submitted in French was translated into English by Doctor Jacques Padawer Department of Biology New York University

References

1 BESSIS M 1954 Traité de cytologie sanguine vol 1 Masson et Cie Paris France

2 BESSIS M & BRETON GORIUS J 1954 Elution des anticorps à partir des cellules osmiquées Compt rend soc biol 148 709-10

3 ANDRE R B DREYFUS & M BESSIS 1954 Anticorps anti leucocytaires dans un cas de leucémie lymphoide associée à une maladie de Hodgkin Rev Hématol 9 50-6

4 BESSIS M & M BRICKA 1952 Aspect dynamique des cellules du sang Son etude par microcinématographie en contraste de phase Rev Hématol 7 407-35

5 BESSIS M & M BRICKA 1950 Etude au microscope électronique sur l'hémolyse l'agglutination la forme et la structure des globules rouges Rev Hématol 5 3-4 396-43

6 POLICARD A & M BESSIS 1953 Fractionnement d'hématies par les leucocytes au cours de leur phagocytose Compt rend soc biol 147 982 83

7 HARGRAVES M M H RICHMOND & R MORTON 1948 Presentation of two bone marrow elements the "tart" cell and the "L.E." cell Proc Staff Meetings Mayo Clinic 23 26-28

8 WILSON A T 1953 The ejection of phagocytized particles by leukocytes J Exptl Med 98 305-10

THE METABOLISM OF HUMAN POLYMORPHONUCLEAR LEUKOCYTES*

By Samuel P Martin,† Gordon R McKinney,‡ and R Green
Department of Medicine Duke University School of Medicine Durham N C

The carbohydrate metabolism of the polymorphonuclear leukocyte has been reviewed recently by Beck and Valentine[1] and will be only briefly summarized because of the completeness of this review The cardinal feature of the metabolism of this cell is its high aerobic lactic acid production which is not influenced by oxygen tension [3] There is an active oxygen consumption and indications that there is an intact Krebs cycle [1, 3] The mature polymorphonuclear leukocyte contains glycogen [4] Wagner and his co workers have shown that this glycogen is utilized to produce reducing and nonreducing intermediates of carbohydrate metabolism by phosphorylase activity [5, 6] Glucose 1 phosphate, fructose 6 phosphate fructose 1 6 diphosphate and phosphoglyceric acid have been isolated [7] The studies isolating these compounds have shown the existence of phosphorylase phosphoglucomutase and phosphohexoisomerase activity and have indicated, as well the presence of triose phosphate dehydrogenase This group has also shown the existence of hexokinase in the insoluble particles of the cell [8]

The present study was undertaken to determine some of the physiological aspects of leukocyte metabolism and to correlate these aspects with reactions in disease Emphasis was placed on the reaction of the intact cell to various environmental changes

Methods

Blood was collected from the cubital vein of various subjects into a non wetting system consisting of either siliconed syringes or a Fenwal blood pack § An ion exchange resin (Dowex 50) in the blood pack or heparin (0 02 mgm /ml) were used as an anticoagulant Sedimentation was accomplished by the use of nonpyrogenic dextran‖ in a final concentration of 1 per cent The platelets white cells and plasma were separated by centrifugation for 15 minutes at 50 × g The resulting sediment contained 90 to 95 per cent polymorphonuclear leukocytes and a red to white cell ratio of from 10 to 1 to as low as 1 to 1 The cells were resuspended in a modification of Hank s solution [3] The concentrations were adjusted to 30 million cells per ml before final study With manometric studies a total of 90 million cells were used The cell number is critical since it influences the rates of respiration and glycolysis The

* Th w k wa ppo t d p t by se h g t f m th N t l I t t t s f H lt[h] [...] t l Stat P bl H lth Se (G t N G3476) th U ted St t At m E gy C mm [...] lC [...] t AT (40 1) 1081] th B t L bo t M t G Ill d th Sp l F dat N w Yo k N Y
† Ma kl S h la M d
‡ P se t dd D p tm t of Ph rm l gy U ty f W t V g S hool f M l M g town W V
§ F al Lab to F m gham M
‖ De t a w supp l d by Ph rm Lt d C mm l Sol t I

cells were placed in a siliconed Warburg vessel or in a 25 ml Erlenmeyer flask with air and shaken at 37 C in a constant temperature waterbath All pipettes used in the procedure were siliconed All solutions were made in nonpyrogenic water When broken cells were used they were homogenized in a ground glass homogenizer with alumina sea sand or without abrasives The presence or absence of abrasives did not alter the cell response

Lactate analyses were carried out by the method of Barker and Summerson[9] on filtrates prepared by the Folin Wu method [10] on fructose by the method of Roe [11] and on glycogen by a composite method [8] Paper chromatographic analysis for lactic acid was carried out by the method of Lugg and Overall [13] Oxygen consumption was measured manometrically Lactic acid was also determined in certain experiments manometrically by measuring the release of CO All values were calculated as micromoles per 10 million cells per hour Ten million cells were used because of the work of Leschel [14] who showed that 10 million leukocytes were equivalent to an approximate weight of one mgm

In a series of experiments with iodoacetate the intact cells were suspended in 3 ml of 0 0025M sodium iodoacetate in modified Hank s solution [8] allowed to incubate at 37° C for 15 minutes then dialysed for eight hours at 10 C against 250 ml of the modified Hank s solution with hourly changes The broken cells were suspended in a 0 066M potassium phosphate buffer (pH 7 4) and dialysed against similar volumes of the same buffer in the manner described above At the end of the dialysis the cells were suspended in a solution of the same buffer used for dialysis and containing a final concentration of 0 01M glutathione (GSH) 0 001M adenosine triphosphate (ATP) 0 003M magnesium chloride and 0 033M glucose or hexosediphosphate In some experiments 0 001M diphosphopyridine nucleotide (DPN) was used These mixtures were incubated for four hours Lactate was determined colorometrically in all experiments and by the manometric and chromatographic methods in representative experiments

Respiration In TABLE 1 the respiration of normal human leukocyte is presented Leukocytes have an active oxygen uptake This uptake diminished in the presence of glucose but not of fructose TABLE 2 shows the effect of Krebs cycle intermediates on respiration in intact and broken cells Values are recorded as plus or minus the per cent change from the control values without the substrate Pyruvate was the only substrate influencing oxygen uptake of intact cells When the cells were injured they responded to a

TABLE 1

OXYGEN CONSUMPTION LACTATE PRODUCTION AND GLYCOGEN UTILIZATION OF HUMAN LEUKOCYTES

(Values expressed as *micromoles per 10 million cell/hour* ± S E)

	QO_2	Q Lactate	Q Glycog
Control	−0 150 ± 0 007	+0 266 ± 0 078	−0 018 ± 0 007
0 0056M Glucose	−0 092 ± 0 006	+0 740 ± 0 107	−0 006 ± 0 003

THE METABOLISM OF HUMAN POLYMORPHONUCLEAR LEUKOCYTES*

By Samuel P Martin,† Gordon R McKinney,‡ and R Green

Department of Medicine Duke University School of Medicine Durham N C

The carbohydrate metabolism of the polymorphonuclear leukocyte has been reviewed recently by Beck and Valentine[1] and will be only briefly summarized because of the completeness of this review The cardinal feature of the metabolism of this cell is its high aerobic lactic acid production, which is not influenced by oxygen tension[2, 3] There is an active oxygen consumption and indications that there is an intact Krebs cycle[1, 3] The mature polymorphonuclear leukocyte contains glycogen[4] Wagner and his co workers have shown that this glycogen is utilized to produce reducing and nonreducing intermediates of carbohydrate metabolism by phosphorylase activity[5, 6] Glucose 1 phosphate fructose 6-phosphate fructose 1 6-diphosphate and phosphoglyceric acid have been isolated[7] The studies isolating these compounds have shown the existence of phosphorylase phosphoglucomutase, and phosphohexoisomerase activity and have indicated as well the presence of triose phosphate dehydrogenase This group has also shown the existence of hexokinase in the insoluble particles of the cell[8]

The present study was undertaken to determine some of the physiological aspects of leukocyte metabolism and to correlate these aspects with reactions in disease Emphasis was placed on the reaction of the intact cell to various environmental changes

Methods

Blood was collected from the cubital vein of various subjects into a non wetting system consisting of either siliconed syringes or a Fenwal blood pack § An ion exchange resin (Dowex 50) in the blood pack or heparin (0 02 mgm /ml) were used as an anticoagulant Sedimentation was accomplished by the use of nonpyrogenic dextran ‖ in a final concentration of 1 per cent The platelets white cells and plasma were separated by centrifugation for 15 minutes at 50 × g The resulting sediment contained 90 to 95 per cent polymorphonuclear leukocytes and a red to white cell ratio of from 10 to 1 to as low as 1 to 1 The cells were resuspended in a modification of Hank s solution[3] The concentrations were adjusted to 30 million cells per ml before final study With manometric studies a total of 90 million cells were used The cell number is critical since it influences the rates of respiration and glycolysis The

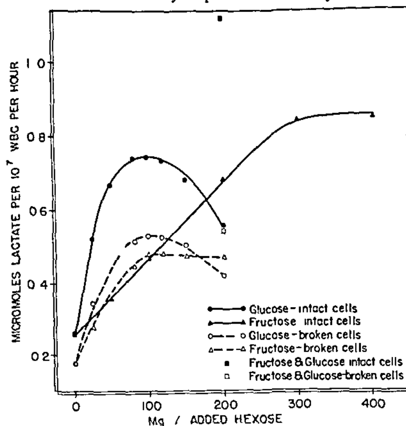
* Th w k w s ppo t d pa t b se b g t f om th N t l I t t t f H lth L t I St t P bl H lth Serv (G t N G34 6) the L t I St t Atom E gy C mm [C t t AT (40 1) 1081] th Ba t L bo t r M to G Ill d th g l F lat New Y k N Y

† M kl S h la M d

‡ P se t dd s D p tm t f Ph m l gy U ty f W t V g S hoo l f M d M g t n W V

§ F w l Labo at r Fr m gh m M

‖ De t w suppli d by Pharmac Ltd and Comm l Sol t I c



F ua 1 The ff t f ry g on trat f gl se d fru t se b lact c d p od t h m le kocyt

hexoses are shown in TABLE 4 The phosphorylated hexoses are not utilized by the intact cell

In TABLE 5 the effect of some intermediates of glycolysis are shown Of these glyceraldehyde 3 phosphate induced the greatest lactate production in the intact cell In TABLE 6 inhibitors of glycolysis are shown Iodoacetate was the most effective inhibitor Oxamic acid an inhibitor of lactic dehydrogenase [16] decreases lactate production by 20 per cent These concentrations will produce marked inhibition of purified lactic dehydrogenase Similar effects were noted under anaerobic conditions Oxamic acid does not affect glyoxylase activity [17] Sorbose 1 phosphate an inhibitor of hexokinase [18] does not influence lactate production in intact or broken leukocytes When added to 0 1M to 0 01M dl glyceraldehyde there was no inhibition of lactate production in fact there was no increase Since dl glyceraldehyde is an inhibitor of triose phosphate dehydrogenase and hexokinase [21] this finding may add to the evidence that the aerobic lactic acid production of the mature poly

TABLE 2

EFFECT OF METABOLITES RELATED TO THE KREBS CYCLE ON RESPIRATION IN INTACT AND BROKEN LEUKOCYTES

(Values expressed as per cent plus or minus control value without intermediate. All determinations represent an average of four or more separate experiments.)

Additions (final Concentration 0.01M except)	Intact	Broken
Pyruvate	+12	+16
Acetate	−2	+7
Oxalacetate	+5	+14
Citrate	−2	+24
alpha Ketoglutarate	+6	+31
Succinate	−3	+108
Fumarate	+4	−11
Malate	−2	−17
Glutamate	−1	+14
Cytochrome *c* (0.00005M)	−3	+17
Succinate + Cytochrome *c*	+4	+129

series of substrates, succinate being the most active. TABLE 3 shows the effect of a series of inhibitors. Malonate does not influence intact leukocytes. The effects with cyanide and other inhibitors are consistent with values reported in the literature. Limited studies on leukocytes from subjects with myelogenous leukemia showed an active respiration comparable to the normal cells.

Glycolysis. In TABLE 1 the lactate production is reported in the presence of air. There was no significant alteration on use of oxygen or nitrogen in normal leukocytes, but an increase was noted when leukemic neutrophilic leukocytes were exposed to nitrogen. This finding confirms earlier observations.[2, 14] Immature neutrophilic leukocytes obtained from individuals with severe infection have normal to increased glycolysis in contrast to the leukocyte of leukemic individuals.

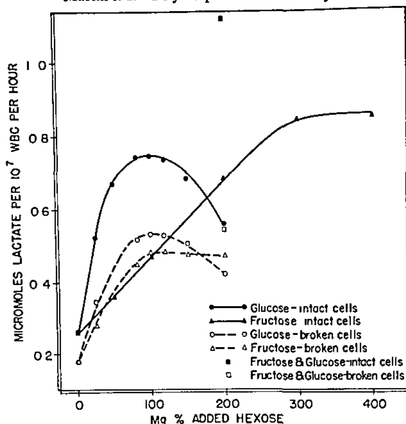
Glycolysis can be abolished by dialysing intact cells. Restoration of Mg and glucose returns lactate production to normal. The effect of various concentrations of glucose and fructose are shown in FIGURE 1. When glucose and fructose are mixed, the lactate yield is additive but fructose utilization is markedly diminished.[15] Quantities of glucose in excess of 0.0056M inhibit glycolysis in the intact cell. The nature of this inhibition is not known. The effects of 0.0056M concentration of glucose, fructose and the phosphorylated

TABLE 3

EFFECT OF INHIBITORS ON RESPIRATION

(See TABLE 2)

Inhibitor	Intact	Broken
Malonate 0.01M	+1	−43
Malonate 0.01M + succinate 0.01	−2	+3
Fluoroacetate	−37	−46
KCN 0.0001M	−81	−75
pChloromercuribenzoate (0.001M)	−85	−83
Dinitrophenol	+36	+41



F 1 Th ff t f arying c trat f gl os d fru t se rb l tic ac d p od t i h m l k yt s [illegible]

hexoses are shown in TABLE 4 The phosphorylated hexoses are not utilized by the intact cell

In TABLE 5 the effect of some intermediates of glycolysis are shown Of these glyceraldehyde 3 phosphate induced the greatest lactate production in the intact cell In TABLE 6 inhibitors of glycolysis are shown Iodoacetate was the most effective inhibitor Oxamic acid an inhibitor of lactic dehydrogenase[16] decreases lactate production by 20 per cent These concentrations will produce marked inhibition of purified lactic dehydrogenase Similar effects were noted under anaerobic conditions Oxamic acid does not affect glyoxylase activity[17] Sorbose 1 phosphate an inhibitor of hexokinase[18] does not influence lactate production in intact or broken leukocytes When added to 0 1M to 0 01M dl glyceraldehyde there was no inhibition of lactate production in fact there was no increase Since dl glyceraldehyde is an inhibitor of triose phosphate dehydrogenase and hexokinase[21] this finding may add to the evidence that the aerobic lactic acid production of the mature poly

may be removed from the compensatory mechanisms of the host and its metabolic alterations studied The cell is morphologically ideal for studies of cell physiology, since suspensions of intact discrete cells are available The cell s fragile nature and its response to injury, however, detract from as well as complicate, studies of it

Acknowledgments

The authors wish to express their thanks to Doctor James Tullis of the University Laboratory of Physical Chemistry, Harvard University and to Doctor Seymour Korkes Doctor Philip Handler, and Doctor R Wayne Rundles of Duke University for their helpful suggestions

References

1 Beck W & W N Valentine 1953 Cancer Research 13 309
2 Kempner W 1939 J Clin Invest 18 291
3 McKinney G R S P Martin R W Rundles & R Green 1953 J Applied Phys 5 335
4 Wagner R 1947 Blood 2 235
5 Wagner R 1950 Arch Biochem 26 123
6 Wagner R 1950 Arch Biochem 29 260
7 Wagner R & A Yourke 1952 Arch Biochem 39 194
8 Wagner R & A Yourke 1953 Arch Biochem 44 415
9 Barker S B & W H Summerson 1941 J Biol Chem 138 535
10 Folin O & H J Wu 1919 J Biol Chem 38 81
11 Somogyi M 1945 J Biol Chem 160 69
12 Umbreit W W R H Burris & J F Stauffer 1949 Manometric Techniques and Tissue Metabolism 191 Burgess Minneapolis Minn
13 Lugg J W H & B T Overall 1948 Australian J Sci Research (A)1 98
14 Peschel E 1930 Klin Wochschr 9 1061
15 Martin S P G R McKinney R Green & C Becker 1953 J Clin Invest 32 1171
16 Hakala M A J Glaid & G W Schwert 1953 Federation Proc 138 535
17 McKinney G In manuscript
18 Lardy H A V D Wiebelhaus & K M Mann 1950 J Biol Chem 187 325
19 Cori G T M W Stein & C F Cori 1948 J Biol Chem 173 605
20 McKinney G R 1953 Arch Biochem 46 246
21 Needham D M L Siminovitch & S M Rapkine 1951 Biochem J 49 113
22 Martin S P S N Chaudhuri R Green & G R McKinney 1954 J Clin Invest 33 358

THE ENZYMES OF LEUKOCYTES*

By William N Valentine

Department of Medicine School of Medicine University of California Medical Center and the Veterans Administration Center Los Angeles Calif

Leukocytes are complex tissues readily biopsied and contain a wide variety of metabolic equipment whose operation as with other tissues reflects the impact of disease With the exception of the nonnucleated erythrocyte they are the most available of all the body cells for repeated analysis and techniques now existent render the study of separated leukocytes readily feasible Their free floating environment in the blood stream and individuality in the tissues do not resemble the structural integration of organs such as liver kidney and muscle which are more conventional tissue sources for enzyme study Never theless it should be recognized that the usual techniques of enzymology are not designed to measure over all organ function but to record the metabolic capacity of some special system in a known amount of tissue cells or tissue homogenates In this respect the leukocytes present many advantages since they can be quantitatively enumerated and differentially counted To be sure, as with all tissues leukocytes must have certain distinctive metabolic characteristics but they also share much of the metabolic machinery common to other body tissues The object of investigations on leukocyte metabolism in this laboratory has been (1) to record descriptively the enzymatic capacities and biochemical content of leukocytes with special reference to the various morphologic leukocyte types (2) to record the effect of disease on these moie ties and (3) to attempt to bring the conventional and established tools of the chemist to bear on clinical problems through the medium of study of the leukocyte

In leukocyte homogenate systems fortified with glucose hexose diphosphate cytochrome C DPN ATP and Mg^{++} and studied by conventional manometric techniques both normal and leukemic cells exhibit a primarily aerobic glycoly tic metabolism[1] The observed ratio of glycolysis to respiration in terms of glucose equivalents has been approximately 30 to 1 in cell homogenates from normal subjects and subjects with chronic myelocytic leukemia and 15 to 1 in those prepared from subjects with chronic lymphocytic leukemia Expressed on the basis of unit cell activity the aerobic over all glycolytic rate is only two fifths as great as normal in homogenates of the leukocytes of chronic myelocytic leukemia and only one sixth normal in similar preparations from chronic lymphocytic leukemia In the latter instance this is partially but by no means entirely due to the fact that the average size of the cell population analyzed is smaller and the unit cell nitrogen content somewhat less than half normal In the case of chronic myelocytic leukemia however unit cell nitrogen content is only slightly below normal These differences in over all glycolytic rate are of course reflected by rather similar changes in glucose

Th t g t w rpo ted by se h g t N H 1060 (C3R) from the N tio l Can I t t t f th N t l In tit te f H lth U t d St t P bl H lth S d by gr t f om P k D d Comp y D t t M h

may be removed from the compensatory mechanisms of the host and its metabolic alterations studied The cell is morphologically ideal for studies of cell physiology, since suspensions of intact discrete cells are available The cell s fragile nature and its response to injury, however detract from, as well as complicate, studies of it

Acknowledgments

The authors wish to express their thanks to Doctor James Tullis of the University Laboratory of Physical Chemistry Harvard University and to Doctor Seymour Korkes Doctor Philip Handler, and Doctor R Wayne Rundles of Duke University for their helpful suggestions

References

1 Beck W & W N Valentine 1953 Cancer Research 13 309
2 Kempner W 1939 J Clin Invest 18 291
3 McKinney G R S P Martin R W Rundles & R Green 1953 J Applied Phys 5 335
4 Wagner R 1947 Blood 2 235
5 Wagner P 1950 Arch Biochem 26 123
6 Wagner P 1950 Arch Biochem 29 260
7 Wagner R & A Yourke 1952 Arch Biochem 39 194
8 Wagner R & A Yourke 1953 Arch Biochem 44 415
9 Barker S B & W H Summerson 1941 J Biol Chem 138 535
10 Folin O & H J Wu 1919 J Biol Chem 38 81
11 Somogyi M 1945 J Biol Chem 160 69
12 Umbreit W W R H Burris & J F Stauffer 1949 Manometric Techniques and Tissue Metabolism 191 Burgess Minneapolis Minn
13 Lugg J W H & B T Overall 1948 Australian J Sci Research (A)1 98
14 Peschel E 1930 Klin Wochschr 9 1061
15 Martin S P G R McKinney R Green & C Becker 1953 J Clin Invest 32 1171
16 Hakala M A J Glaid & G W Schwert 1953 Federation Proc 138 535
17 McKinney C In manuscript
18 Lardy H A N D Wiebelhaus & K M Mann 1950 J Biol Chem 187 325
19 Cori G T M W Stein & C F Cori 1948 J Biol Chem 173 605
20 McKinney G R 1953 Arch Biochem 46 246
21 Needham D M L Siminovitch & S M Rapkine 1951 Biochem J 49 113
22 Martin S P S N Chaudhuri R Green & G R McKinney 1954 J Clin Invest 33 358

leukemia morphologically, mean myeloid leukocyte glycogen is half again the normal mean and three times that of myelocytic leukemia[3] Leukocyte glycogen must of necessity depend upon the forces favoring synthesis and those favoring utilization or degradation but these metabolic differences remain unexplained in terms of differences in enzyme machinery in the two situations Since blast cells and lymphocytes contain little or no glycogen, analyses of leukemic cell populations composed predominantly of these types reveal very low unit cell glycogen

Attention has been directed to certain nonenzymatic leukocyte constituents of biological importance as a corollary to studies on the enzymatic constitution of leukocytes These constituents include histamine free sulfhydryl compounds (free glutathione), and glucuronic acid Histamine is confined entirely or almost entirely to the myeloid series of leukocytes[4] and reaches extremely high levels in chronic myelocytic leukemia[5, 6, 7, 8] Available data fail to indicate that the eosinophil has any special importance in histamine metabolism[7, 9] and until recently attempts to correlate histamine levels with the basophil had not been successful. Recently Ehrlich[10] suggested on the basis of reanalysis of some few cases from our early data that there was a better correlation of blood histamine with absolute basophil counts than with absolute neutrophilic granulocyte counts The opportunity has just presented itself to study repeatedly the blood histamine in a patient with chronic myelocytic leukemia in whom 40 to ,0 per cent of the leukocytes were basophils At a leukocyte level of 80 000 per cu mm histamine per ml of blood was nearly double that of any other case studied Even after therapy had brought the total leukocyte count to essentially normal (11 400 per cu mm but with 67 per cent basophils) the blood histamine was 85 times the upper limits of normal values This finding prompted re evaluation of 22 studies of blood histamine in chronic myelocytic leukemia[11] The evidence very strongly suggests that the basophil is the chief or sole carrier of histamine in the blood of man FIGURE 2 indicates this relationship of basophils to leukocyte histamine in chronic myelocytic leukemia

The content of free reduced glutathione (GSH) has also been studied in this laboratory[12] in leukocytes and erythrocytes in a number of hematologic disorders On a cell for cell basis the leukocyte was found to contain 7 times as much free GSH as the erythrocyte Although GSH has been suggestively implicated in cell growth and in neoplastic processes we were unable to detect any clear cut relationship of free sulfhydryl to either increased myelopoietic or erythropoietic activity in a variety of nonleukemic states nor were elevated GSH values found in leukemia In fact the unit cell GSH content in blastic and lymphocytic leukemia appeared consistently low Leukocytes also contain readily measurable free glucuronic[13] acid averaging slightly above 3 5 mgm per 10^{10} leukocytes in normal subjects Leukemic blast cells and lymphocytes as well as the lymphocytes of nonleukemic lymphocytoses (such as for example infectious mononucleosis) are poor in glucuronic acid

Studies have also been conducted on the esterase[14] beta glucuronidase[15] and on the acid and alkaline phosphatase of leukocytes[16, 17, 18] under a variety of clinical circumstances Detailed discussion of these appear elsewhere but

utilization The glucose in the medium is measured as utilized in these experiments when it has undergone the first phorphorylation to glucose 6-phosphate Oxygen consumption is similarly much lower in leukemia than in normal homogenates on a unit cell basis Doctor William Beck has recently been engaged in assaying individually many of the enzymes and cofactors of the Embden Myerhoff cycle in samples of normal and leukemic cells with the end in view of possibly finding a rate limiting step responsible for the diminshed glycolytic activity of leukemic homogenates[2] Interestingly, while the aldolase activity and the amount of total pyridine nucleotides were essentially normal in the cells of chronic myelocytic leukemia, the lactic dehydrogenase and triose phosphate dehydrogenase were reduced approximately proportional to the differences in over all glycolysis FIGURE 1 records the comparison of these constituents and enzymatic activities in normal subjects and in chronic myelocytic leukemia At first, these activities seemed possible rate limiting steps in glycolysis in chronic myelocytic leukemia but studies on the capacities of these enzymes indicate that while reduced in amount, they still far exceed the over all glycolytic rate

Myeloid leukocytes contain substantial amounts of glycogen which are not significantly altered from normal in the presence of either uncontrolled diabetes or adrenal steroid therapy[3] However in chronic myelocytic leukemia, the myeloid leukocyte glycogen content averages only half normal, while in polycythemia rubra vera with leukemoid features closely simulating myelocytic

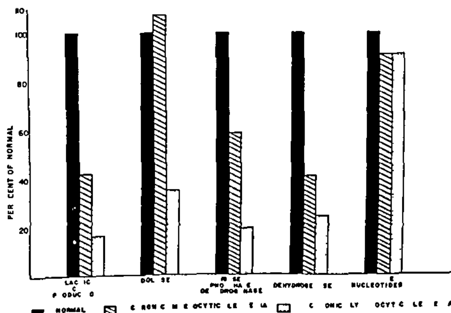
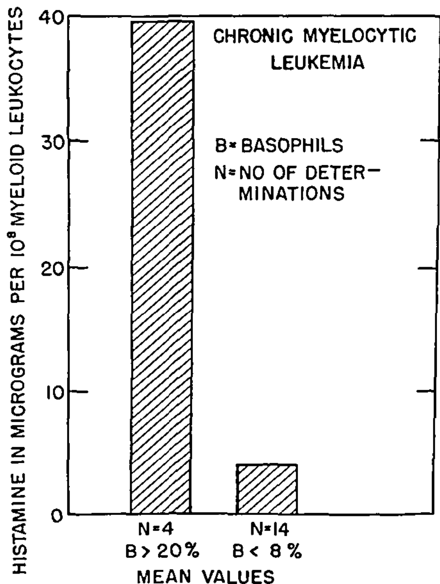


FIGURE 1 Compariso[n] [illegible] leukocyte homogenates [illegible] The m[illegible] is [illegible] 100 per c[ent] [illegible] lymphocyt[ic] leukem[ia] [illegible] per cent [illegible]

health Expressed as mgm of phosphorus liberated from substrate per hour per 10^{10} leukocytes the normal mean of our original studies was 25 and the range 13 to 58 With continuing experience this finding has continued to be essentially the case though occasional normal individuals have been observed with values lower than 13 In chronic myelocytic leukemias (and in fact in other leukemias as well) values have been uniformly low with a mean of *only 3 0 This decrease in itself might theoretically be due to the presence of* less mature cells in the analytical system but in polycythemia vera with leukemoid features and cell populations morphologically indistinguishable from those of chronic myelocytic leukemia the mean value has been 20 times greater than that of the leukemic cells [18] There has been no observed overlap and mean values have been three times greater than normal Similar findings are observed in certain myeloproliferative syndromes resembling chronic myelocytic leukemia but are thought to be neither the latter nor polycythemia vera In the initial investigations it was observed that very great elevations in unit leukocyte alkaline phosphatase occurred in the presence of infections and in certain other causes of neutrophilic leukocytosis Since on the average these elevations were increased four to five times above normal and often were individually greater the increase could not be explained by morphological deviations from the normal cell population in the analytical systems Similar qualitative increases in leukocyte phosphatase have been observed in polycythemia vera and in infection by cytochemical staining of blood films for alkaline phosphatase [19] Wider experience with the phenomenon has demonstrated that it accompanies many situations in which stress or increased pituitary and/or adrenal cortical activity would be expected to be present Thus consistent marked elevations in unit leukocyte alkaline phosphatase are observed not only in the presence of infection but also in association with myocardial infarction bleeding aneurysms ruptured peptic ulcer fractures cerebral vascular accidents, acute urinary retention operative procedures of various types and pregnancy [20] In infection and many other disorders this elevation continues to be present during convalescence for a time even when therapy has restored the total and differential leukocyte count to the normal range Elevated leukocyte alkaline phosphatase has been observed in cases of active pulmonary tuberculosis in the absence of any gross abnormality of total or differential leukocyte counts In nine patients studied before and after surgical procedures the leukocyte alkaline pho phatase rose stepwise after operation attaining a peak of about three times baseline values on the average 72 hours after the procedure In the absence of complications a fall toward normal occurred during the next several days In the presence of complications values remained elevated or rose still higher FIGURE 3 records the observations in two patients with unusually marked responses Of considerable interest were observations that this ubiquitous reaction did not occur except rarely in certain situations which might *a priori* be considered stresses The reaction was a rare accompaniment of active uncomplicated rheumatoid arthritis even in the presence of pain swollen joints and elevated



FIGU E 2 Cor p so f h stam t t m g m per 10 ell i h o myelocyt le k m de rc m t c wh (1) th b soph l pe entage ded 20 pe t d (2) th b soph l pe e tage wa bel w 8 pe c t

recent observations on the variations in alkaline phosphatase activity of leukocytes in disease are of interest Alkaline phosphatase was measured as previously described[16] at pH 9 9 and 37° C employing sodium beta glycerophosphate as substrate and a 1 hour incubation period It has proved to be an enzyme responsive in a most interesting way to deviations from normal

the same metabolic response as those discussed above despite the serious nature of the illnesses

The above observations have naturally led to exploration of the role of pituitary adrenal activity in this phenomenon[20] When 30 to 40 units of ACTH gel* are given intramuscularly every eight hours for three days to subjects under no apparent stress and with normal leukocyte alkaline phosphatase values there regularly occurs a marked rise in *unit cell* leukocyte alkaline phosphatase This rise reaches a peak averaging four times greater than pretreatment levels by the end of 72 hours Seventy two hours after cessation of treatment values are entirely normal Although substantial neutrophilic leukocytosis occurs the peak alkaline phosphatase and peak leukocytosis do not coincide Values of course are compared on a *unit cell* basis Only relatively slight and inconsistent elevations presumably due to the intramus cular protein injection occur when the gelatin medium without ACTH is given in equal amounts for a similar period of time The phenomenon is produced therefore both by the presence of stress continuing over a con siderable interval of time and by repeated injections of ACTH gel Further, it does not resemble the eosinopenic response which develops maximally within four to six hours of a burst of pituitary adrenal activity

The mechanism of the response is not clearly delineated † Cortisone acetate and hydrocortisone in doses up to 200 mgm daily fail to duplicate the phenom enon fully In some cases a moderate rise occurs but this rise quickly levels off and may in some instances actually appear to be returning in the direction of normal before the three-day experimental period is terminated and never attains the high levels reached when ACTH gel is administered or when certain types of natural stresses such as infection are present Experience with aqueous ACTH is too scanty at present for any conclusions but initial results have been much more variable than with the purified ACTH gel FIGURE 4 shows the characteristic response to ACTH gel administration in a representa tive patient Response of the same patient to injection of the gelatin medium alone and to cortisone acetate and hydrocortisone are also shown At the present time it is not entirely clear whether the observed phenomenon is due to some steroid elaboration of the adrenals or to a combination of adrenal steroids, whether some direct pituitary activity independent of the adrenals is present or whether some combination of the above with tissue injury is playing a dominant role Elevations of alkaline phosphatase activity in rat liver have been observed after adrenal steroid administration by Kochakian and Bartlett[21] and the relationship of the adrenals to phosphorylation and to alkaline phos phatase has been reviewed by Vérzar[22] The phenomena may not apply to all species since we have not been able to reproduce it in dogs experimentally The meaning of the rather remarkable changes in unit cell alkaline phosphatase activity under the natural and experimental conditions discussed above and the implications of its possible relationship to pituitary adrenal activity remain

* Co t ph O ga

† Prel mi ry d ta w th lo ger se l ACTH g l gge t th t w th t d dm tr t l k cyt lk l phosph tase m y ret rn to rm l wh l pat t the m d t I th re pe t th ACTH d ced le ti may diff f m th t d d by f t tr m f

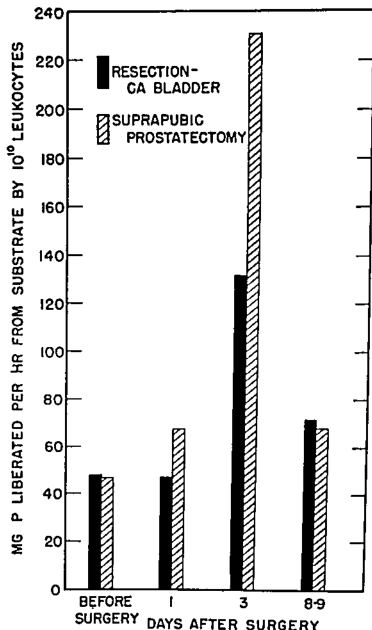


FIGURE 3 Un t leukocyt lkal phosphatase l tw s b t befo e a d fte s g c l p oc d re

sedimentation rates It was not observed in any of eight cases of active disseminated lupus erythematosus and was absent (though less consistently so) in cases of acute rheumatic fever without streptococcal complications or bacterial endocarditis These groups of disorders apparently failed to elicit

4 CODE C F 1937 The source in blood of the histaminelike constituent J Physiol 90 349
5 CODE C F & A D McDONALD 1937 The histaminelike activity of blood Lancet 2 730
6 THIERSCH J B 1947 Histamine and histaminase in chronic myeloid leukemia of man Australian J Exptl Biol Med Sci 25 73
7 VALENTINE W N M L PEARCE & J S LAWRENCE 1950 Studies on the histamine content of blood with special reference to leukemia leukemoid reactions and leukocytoses Blood 5 623
8 SHIMKIN M B L SAPIRSTEIN F R GOETZL, P M WHEELER & N I BERLIN 1949 Blood histamine in leukemia and erythremia J Natl Cancer Inst 9 379
9 CODE C F 1953 The histamine content of white blood cells Blood cells and Plasma Proteins 292–305 J L Tullis Ed Academic Press New York N Y
10 EHRICH W E 1953 Histamine in tissue mast cells Science 118 603
11 VALENTINE W N J S LAWRENCE M L PEARCE & W S BECK The relationship of the basophil to blood histamine in man Blood In press
12 HARDIN E B W N VALENTINE J H FOLLETTE & J S LAWRENCE 1954 Studies on the sulfhydryl content of human leukocytes and erythrocytes Am J Med Sci 228 73
13 FOLLETTE J H W N VALENTINE E B HARDIN & J S LAWRENCE 1954 Studies on the glucuronic acid content of human leukocytes in health and disease J Lab Clin Med 43 134
14 Unpublished data
15 FOLLETTE J H W N VALENTINE & J S LAWRENCE 1952 The beta glucuronidase content of human leukocytes in health and disease J Lab Clin Med 40 825
16 VALENTINE W N & W S BECK 1951 Biochemical studies on leucocytes I Phosphatase activity in health leucocytosis and myelocytic leucemia J Lab Clin Med 38 39
17 BECK W S & W N VALENTINE 1951 Biochemical studies on leukocytes II Phosphatase activity in chronic lymphatic leukemia acute leukemia and miscellaneous hematologic conditions J Lab Clin Med 38 245
18 VALENTINE W N W S BECK J H FOLLETTE H MILLS & J S LAWRENCE 1952 Biochemical studies in chronic myelocytic leukemia polycythemia vera and other idiopathic myeloproliferative disorders Blood 7 959
19 WACHSTEIN M 1946 Alkaline phosphatase activity in blood and bone marrow cells J Lab Clin Med 31 1
20 VALENTINE W N J H FOLLETTE E B HARDIN W S BECK & J S LAWRENCE 1954 Studies on leukocyte alkaline phosphatase activity Relation to stress and pituitary adrenal activity J Lab Clin Med 44 219
21 KOCHAKIAN C D & M N BARTLETT 1948 The effect of crystalline adrenal cortical steroids dl thyroxine and epinephrin on the alkaline and acid phosphatase and arginase of the liver and kidney of the normal adult rat J Biol Chem 176 243
22 VERZAR F 1953 Adrenal cortex and carbohydrate phosphorylation 152–165 Colloquia on Endocrinology Churchill London England

SUBJECT S

ACTH GEL 40 UNITS Q 8 HRS

HYDROCORTISONE 50 MG Q 6 HRS

GEL ALONE 1cc Q 8 HRS

CORTISONE 75 MG Q 6HRS

CORTISONE IM 65 MG Q 8 HRS

MEDICATION

MG P LIBERATED PER HR FROM SUBSTRATE BY 10^{10} LEUKOCYTES

0 20 40 60 80 100 120

BEFORE Rx 24 48 72

TIME IN HOURS

FIGURE 4 Effe t f ACTH i g l t m d um th gelat med m l c rt so a tate d hyd ocor t so (Lo t f) t ell l ukocyt lk l ph phatase r se tat p t t Th ACTH gel nd g lat med um r g n t am sc larly Co t son w g nt muscula ly d gnated e ta ce d lly n a oth Hyd oc t so wa dm t d lly Med c t re g e nd dually i ch se ft pe od whe p t nt w n m d t

obscure Its possible usefulness as an index of such activity also remains to be explored *

References

1 BECK W S & W N VALENTINE 1952 The aerobic carbohydrate metabolism in health and leukemia I Glycolysis and respiration Cancer Research 12 818

2 BECK W S & W N VALENTINE 1954 The glycolytic rate and its relationship to certain glycolytic enzymes and coenzymes in normal and leukemic leukocytes Proc Am Assoc Cancer Research (April)

3 VALENTINE W N J H FOLLETTE & J S LAWRENCE 1953 The glycogen content of human leukocytes in health and disease J Clin Invest 32 251

Th mat l p se ted by the tho r p se t llabo t t d w th h lle gue Doct r J m s H F ll tt Doct r W ll am S B ck Doctor E B t Ha d a d Doct J h S L w

All substrates used in this study were commercial preparations The progress of peptide hydrolysis was followed at 38 C by the colorimetric ninhydrin method as described for serum peptidases [2] The results are expressed either in terms of p the fractions of substrate which were hydrolyzed during the time of enzymatic reaction or whenever the kinetics of a reaction under one set of conditions has been established in terms of θ defined as the initial hydrolysis rates per minute per 10^{10} cells in 1 ml of reaction mixture These activities refer to peptide concentrations of 5×10^{-3} molar For the determination of θ in different bloods under standard conditions two samples were taken during the enzymatic reaction at two and four hours and the mean activity was determined As a rule the two values were in good agreement

In leukocytes from normal blood the contribution of the erythrocytic peptidases to the over all activity was usually less than 5 per cent and was therefore disregarded In leukocytic preparations from some pathologic bloods this fraction may be increased to 10 per cent or even higher, and corrections should then be applied

Results and Comment

The hydrolysis of glycylglycine by leukocytes from normal blood In the absence of an activating ion glycylglycine is split at a relatively slow rate by leukocyte preparations The addition of various ions to the reaction mixture at three different concentrations gave results which are summarized in TABLE 1 It is seen that cobalt alone functions as an activator while zinc, cadmium and bivalent copper appear to have an inhibitory effect The cobalt activation was studied in greater detail In either phosphate or veronal buffer of pH 7 7 increasing the concentration of this ion to 10^{-4} molar caused an increase in the rate of hydrolysis of glycylglycine At still higher concentrations the activity decreased sharply in veronal buffer until at 10^{-3} molar Co^{++} it was no greater than in its absence In phosphate buffer however a plateau was observed presumably because the concentration of the free ion cannot be increased

TABLE 1
EFFECT OF METAL IONS ON GLYCYLGLYCINE HYDROLYSIS BY NORMAL LEUKOCYTES
(pH 7 7 8.3 × 10 cells per ml time 6 hr)

Io	p		
	10 m l	10 m la	10^{-3} mol
None		15	
Co^{++}	45	70	66
Ni^{++}	09	13	07
Fe^{++}	12	12	08
Fe^{+++}	10	12	10
Mn^{++}	11	13	12
Zn^{++}	09	05	03
Cd^{++}	04	03	05
Cu^{++}	12	08	01

PEPTIDASES IN HUMAN LEUKOCYTES*

By Gerard A Fleisher†

Section of Biochemistry Mayo Clinic and Mayo Foundation ‡ Rochester Minn

The prominent functions of the leukocytes in the defense of the body against invasion by microorganisms aroused the interest of early investigators in the protein catabolism of these cells (see bibliography of Merten and Winschuh[1]) Unfortunately the difficulty of separating leukocytes from erythrocytes with out impairing their structural integrity, the instability of some of the peptidases and the lack of adequate analytic methods have greatly impeded progress in this field In recent years these difficulties have been largely overcome Leukocytes may now be separated in viable form from the bulk of the erythrocytes by a variety of methods, and satisfactory procedures are available for the analysis of enzymatic protein degradation and peptide hydrolysis

The present study has been confined to the enzymatic cleavage of a small number of dipeptides and tripeptides by human leukocytes Initially, the cells were obtained from a few selected healthy persons whose total leukocyte count was rather high so that the characteristics of peptidase reactions could be determined with a minimum of blood Special emphasis was laid on the investigation of pH curves activating and inactivating effects particularly of heavy metal ions kinetics influence of buffers and stability of the enzymes An elaboration of these characteristics served three purposes first of all, to establish the optimal conditions for a routine assay of normal and pathologic bloods second, to obtain indications as to which peptides are hydrolyzed by the same enzyme, and last but not least to allow comparisons to be made between the corresponding enzyme patterns in serum[2 3] erythrocytes,[4] and tissues[5] The source of serum peptidases, for instance has not been proved as yet While some observations suggested that it might be the lymphocytes[6 7] correlations between the cell count and some serum peptidase activities under conditions of induced stress have certainly not been established,[8 9] on the other hand serum peptidases may be significantly elevated in some cases of lymphocytosis[5]

Experimental

The blood leukocytes were isolated by a modification of the method of Buckley and his co-workers[10] The final preparations when stained with Wright s stain yielded differential counts which were nearly identical with those taken on the original blood films The lymphocyte preparations were obtained from surgical specimens of enlarged lymph nodes The greater part (about 75 per cent) consisted of small and medium sized lymphocytes There was only about 1 per cent of polymorphonuclear cells the remainder being made up of broken cells presumably also lymphocytes The details of the isolation procedures will be described elsewhere

Th s contribut is paper No 3 the se es Pept dase in Human Blood
† With th tech cal a sistance f J Ha ge
‡ Th M y Foundat n i a p rt of th Graduate School of the U rs ty of M n sota Rochest r M n

sion was reached from the observation that an ultracentrifugate (35 000 × g) of an isotonic dilution of previously frozen cells contained about 88 per cent of the activity of another aliquot of the same cells which had been frozen and lyzed with water but not centrifuged The experiment showed at the same time that freezing alone causes cytolysis of the majority of cells A study of the kinetics of the glycylglycine hydrolysis under optimal conditions revealed the parameter r = 1 0 that is a reaction halfway between first order and zero order The average activity and standard deviation in the cells from 25 normal persons was determined to be $\theta = 37 \pm 10$

It has been shown[2] that the hydrolysis of glycylglycine by human serum is activated more by manganese than by cobalt ion For this reason the effect of Mn^{++} on leukocyte preparations was reinvestigated under conditions which had proved most favorable for the testing of the serum enzyme namely veronal buffer of pH 7 4 and high concentrations of Mn^{++} All ions normally present in serum were also added to the reaction mixture Of the different concentrations of Mn^{++} tested 2×10^{-3} molar proved to be the optimum just as in the case of serum However with leukocytes the activity was only about one fifth of the optimal activity in the presence of Co^{++} provided the same buffer was used It is not known whether the two ions activate the same enzyme

The hydrolysis of glycylglycine by lymphocytes The cell preparations obtained from lymph nodes showed the same behavior as blood leukocytes with respect to pH-optimum maximal cobalt concentration lack of a peak at pH 6 8 and lower activity in veronal buffer as compared with phosphate buffer However per unit cell the activity of lymphocytes was noted to be considerably higher than that of normal blood leukocytes namely $\theta = 70$ and 71 in two different preparations Similar results were obtained on the leukocytes isolated from the blood of two patients with a high degree of lymphocytosis In one of these the differential lymphocyte count of 88 per cent was caused by infectious mononucleosis the leukocytic glycylglycine dipeptidase had the activity $\theta = 74$ The other patient was a 2 year-old boy in whom the cause of the lymphocytosis was not known His blood leukocytes contained 77 per cent lymphocytes with an activity $\theta = 64$

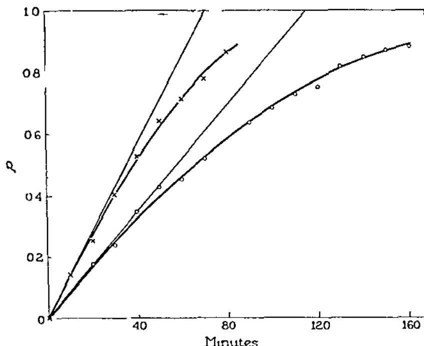
The hydrolysis of other dipeptides by leukocytes from normal blood A number of other dipeptides were used as substrates for leukocytic peptidases Those containing leucine and several other of the heavier amino acids were found to be split by a separate enzyme which will be the subject of another publication The results on the hydrolysis of some lighter peptides in the absence and presence of cobalt ion are summarized in TABLE 2 While racemic preparations were used there was no indication of any significant hydrolysis of the unnatural D forms On the other hand the possibility of competitive inhibition of the hydrolysis of the L-peptides by their D isomers must be kept *in mind* The figures show that only glycyl L-serine is activated by Co^{++} though to a lesser degree than glycylglycine but it is not possible to say at this time whether the two substances are split by the same enzyme The hydrolysis of glycyl L-alanine is not affected by Co^{++} that of L-alanylglycine and glycyl L-valine is actually inhibited This fact makes it quite convincing that these three peptides are not attacked by the glycylglycine dipeptidase but by different

FIGURE 1 Eff t f v ry g pH on th hyd ly f glycylgly i (GG) d glycylgl cylgly (CGG) by ormal l kocytes ● GG with 10 m la Co^{++} 8 1 × 10 ll per ml t m 6 h o GCG 1 6 × 10 ells pe ml time 8 h

beyond about 10^{-4} molar owing to the formation of cobalt phosphate Under optimal conditions the activity in veronal buffer was considerably less than in phosphate buffer

The variability of the enzyme activity with pH in the presence of optimal cobalt concentration is shown in FIGURE 1 A single maximum occurs at about pH 7 7 This curve differs characteristically from that obtained with serum [2] which is made up of three peaks at pH 6 2 6 8 and 8 0 The absence in leukocytes of a cobalt sensitive activity with optimum at pH 6 8 was further substantiated when an enzyme preparation was incubated with Co^{++} for four hours without loss of activity Such experiments when extended to the region of pH 7 7 also proved that the activation by Co^{++} does not require time

The glycylglycine splitting enzyme is not very stable When a solution of leukocytes buffered at pH 6 3 was allowed to stand at 4 C for two days more than half of the original activity was lost apparently by an irreversible process Almost inactive preparations were obtained after incubation for six hours at 38 C followed by dialysis at 4 C The glycylglycine dipeptidase appears to be located in the soluble part of the leukocytes This conclu



FIGU 2 K t cs f th leukocyt tripeptidase react (GGG) o w th 10^{-3} m la Co^{++} both t pH 7 0 w th 7 8 X 10 lls pe ml

To test whether the tripeptidase contains a metal ion or other coenzyme a leukocyte preparation was dialyzed at pH 3 8 and 25° C for three hours The dialyzable portion was then concentrated *in vacuo* and recombined with part of the dialyzed enzyme The other part was tested by itself The result showed that 85 per cent of the original activity was destroyed and could not be recovered by adding the low molecular fraction In this respect, the leukocytic tripeptidase seems to differ from the tripeptidase in duodenal mucosa of the hog[14] In another similar experiment it was seen that Co^{++} is also unable to effect reactivation Subsequently this problem was attacked with the help of the chelating agent ethylenediamine tetraacetic acid (EDTA) The inactivation of the tripeptidase by EDTA depended on the time of contact as well as on the pH as is shown in FIGURE 3a The effect of EDTA on the enzyme during the hydrolysis of glycylglycylglycine is of course not known While it is conceivable that the peptide exerts some protective effect such an assumption has not been made in the plot FIGURE 3b shows how the inactivation at pH 7 0 depends on the concentration of EDTA only within a narrow range At concentrations of EDTA in excess of 10^{-4} molar the inactivation remains constant These findings seem to indicate that active tripeptidase is a metal complex which reacts with hydrogen ion but its equilibrium in the absence of EDTA is very much in favor of the metal complex

TABLE 2
HYDROLYSIS OF VARIOUS DIPEPTIDES BY NORMAL LEUKOCYTES
(pH 7 7 1.5 × 10^{6} cells per ml)

	Ion					
	None			10^{4} molar Co^{++}		
	2 hr	4 hr	10 hr	2 hr	4 hr	10 hr
	p					
Glycylglycine	09	12	29	51	73	1 00
Alanylglycine	94	95	1 00	46	78	88
Glycylalanine	56	79	98	58	78	1 00
Glycylserine	30	48	80	66	92	1 00
Glycylvaline	80	89	1 00	46	76	91
Acetylglycine	02	03	07	02	04	09

Reaction time

enzymes The high rate of the hydrolysis of L-alanylglycine already observed by Husfeldt in 1931 [11] is noteworthy Acetylglycine which lacks the free amino group of glycylglycine is split very slowly, and Co^{++} has little if any effect These results conform to the findings on the high substrate specificity of the glycylglycine dipeptidase from rat muscle and human uterus [12]

Tripeptidase activity in leukocytes from normal blood This enzyme hydrolyzes one of the two peptide bonds of glycylglycylglycine and presumably of other tripeptides as well It has previously been reported[13] to be present in the buffy coat obtained by centrifugation of blood in constricted tubes The enzyme was tested in the presence of cobalt ion and a significantly higher activity was claimed to occur in the cells of old people In the present study, cobalt was not used routinely since the tripeptidase is highly active by itself, and since Co^{++} activates the glycylglycine dipeptidase this makes the tripeptidase reaction difficult to evaluate particularly in cases in which the tripeptidase activity is low and the dipeptidase activity high In order to determine whether cobalt has an activating effect on the tripeptidase, initial reaction velocities should be determined in the presence and absence of this ion This procedure was followed in the reactions shown in FIGURE 2 It can be seen that, under the conditions of the experiment Co^{++} caused about 60 per cent activation In the absence of Co^{++} the reaction kinetics had a parameter r = 2 4 In the presence of Co^{++} the kinetics appeared to have somewhat more zero-order characteristics but this finding was probably due to the superimposition of the dipeptidase reaction The optimal pH was determined to be 7 0 as shown in FIGURE 1 This pH curve is very similar to the curves obtained with serum[3] and erythrocytes[4] but differs from the curves obtained with human tissues such as liver and intestinal mucosa[5] which have pH optima between 7 6 and 7 8 It was thought that the shape of the pH-curve of the leukocytic enzyme might perhaps indicate the presence of smaller amounts of an alkaline tripeptidase Apparently this is not the case as will be shown later

are capable of a direct activating effect on the enzyme or only replace one active ion from its complex with EDTA cannot be decided at this time

It should be mentioned that preincubation of the inactive enzyme with the metal ions did not produce any additional activation This reaction shows that the activation by metal ion proceeds at a much faster rate than the inactivation by hydrogen ion

In an attempt to determine the nature of the endogenous metal activator leukocyte preparations were inactivated almost completely with EDTA at pH 6 3, followed by thorough dialysis against the same buffer at 4° C The result was entirely unexpected for it showed that the dialysis had completely reversed the inactivation by EDTA Apparently the metal complex of EDTA is not in a form which can pass through a cellophane membrane though it must be in equilibrium with free EDTA

Because such preparations were nearly free from interfering dipeptidases they have been found particularly useful for studying some properties of the tripeptidase The activation by cobalt, for instance could be confirmed Such preparations also showed strong activity with two other tripeptides L-leucylglycylglycine and D,L alanyl gylcylglycine In the latter case only the L-form was hydrolyzed Both reactions came to a standstill after one bond had been broken The kinetics of the hydrolysis of these two substrates showed much more zero order characteristics than that of glycylglycylglycine so that algebraic functions may be used up to at least 70 per cent hydrolysis without introducing a significant error The rates for L leucylglycylglycine and L alanylglycylglycine were respectively about 40 and 120 per cent greater than for glycylglycylglycine The relatively higher rate of L alanylglycyl glycine as compared with that of L leucylglycylglycine was already noted by Husfeldt [11] L-leucylglycylglycine is particularly well suited as a substrate to determine which of the two bonds is attacked by the enzyme Paper chromatography of the product of the tripeptidase reaction showed the presence of glycylglycine The other constituent could not be identified since leucine and leucylglycine traveled at the same rate This result indicates that it is the bond nearest the free amino group which is hydrolyzed * The tripeptidase like the glycylglycine dipeptidase is found predominantly in the soluble fraction of an ultracentrifugate regardless of whether cytolysis has been effected by freezing alone or in combination with hypotonicity

With glycylglycylglycine as the substrate the average activity and standard deviation in the cells from 25 normal persons was 151 ± 36 This group includes three healthy men 61 71 and 79 years of age whose activities were not significantly different from those of younger people Likewise there was no greater activation by added Co^{++} in the older than in the younger age group

Tripeptidase activity in lymphocytes In lymphocytes isolated from lymph nodes the tripeptidase had the same pH optimum but the activity was very much lower than in the average leukocytes from normal blood The two lymphocyte preparations showed these activities $\theta = 37$ and 44 The activity was similarly low in the leukocytes isolated from the blood of the patients

I m d bt d t Doct E Fl k f th d term tio

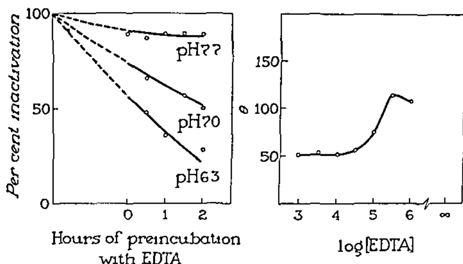


FIGURE 3 Effect of EDTA th l ukocyt trip pt d e r act on ((l ft)] Time of pre cubation with EDTA v d [b (ight)] Con nt at f EDTA t pH 7 0 var ed, p ncubated 2 h All w th 2 8 × 10 cells per ml react on tim 4 hr

It could not be excluded *a priori* that the pH-dependence of the EDTA inactivation is a reflection of the presence of two tripeptidases with different susceptibilities to this chemical A leukocyte preparation was therefore partially inactivated with EDTA at pH 6 3 aliquots were then adjusted to different pH values and the tripeptidase activity was measured The resulting pH curve of the remaining activity was similar to that of the initial activity, and it thus indicated the presence of only a single tripeptidase in leukocytes

The EDTA inactivated tripeptidase *may be reactivated by* Co^{++} Zn^{++} *or* Mn^{++} provided these ions are present in concentrations of at least 10^{-4} molar (TABLE 3) the greater effect of Co^{++} is clearly seen Whether all three ions

TABLE 3

INACTIVATION OF TRIPEPTIDASE (GLYCYLGLYCYLGLYCINE) BY EDTA AND REACTIVATION BY METAL IONS

(pH 6 3 3 9 × 10^5 cells per ml time 4 hrs All concentrations refer to the final mixtures)

EDTA	I †	p		
		10 m l r	10^{-} m la	10^{-4} m la
0	None		78	
10^{-4}	None		05	
10^{-4}	Co^{++}	05	84	96
10^{-4}	Zn^{++}	07	[illegible]9	59
10^{-4}	Mn^{++}	04	64	60

Pre cubated 6 h at 38 C
† Not pr incubated

Discussion of the Paper

MYRON TANNENBAUM (*New York University New York N Y*) A knowledge of the mechanisms of the synthesis of proteins or their precursors should prove important in the solution of problems relating to the biochemistry of blood formation in health and disease Until very recently the proteases or endopeptidases and the peptidases or exopeptidases have been assayed for and studied from a hydrolytic approach Bergmann has suggested that these enzymes might catalyze protein and peptide formation *in vivo* by mass action It has been shown by Fruton and his associates that peptide synthesis can occur by transamidation and transpeptidation reactions In the light of these findings it might be profitable now to examine the kinetics mechanisms of inhibition and the relation of carbohydrate and fat metabolism to the synthetic actions of the peptidases

In our own studies we have detected the hydrolysis of glycylglycine and glycylglycylglycine by rat bone marrow The proteolytic coefficient or the rate of hydrolysis of these synthetic substrates is very large for bone marrow as compared to other tissues *e g* plasma and blood cells It may well be that these enzymes assist in the synthesis of blood cell proteins

with exceptionally high lymphocyte counts For the first patient, suffering from infectious mononucleosis, the activity was $\theta = 47$, for the second $\theta = 88$ It could be concluded that the low tripeptidase activity of lymphocytes is due to lack of cobalt since there is no significant difference in the activation by Co^{++} between these cells and the normal leukocyte mixtures

Summary

(1) Leukocytes contain a glycylglycine dipeptidase which is activated specifically by cobalt ion and shows optimal activity at pH 7 7 Lymphocytes appear to be significantly more active than polymorphonuclear leukocytes The glycylglycine splitting activities of serum with optima at pH 6 2 *and 6 8 are not seen in leukocytes*

(2) Several other dipeptides are split by preparations of leukocytes but different enzymes are most probably involved in these reactions

(3) Leukocytes contain also a tripeptidase which hydrolyzes various tripeptides at the bonds adjacent to the amino group This enzyme is more stable than the glycylglycine dipeptidase With glycylglycylglycine it has an optimum at pH 7 0 It is a metal protein and is activated somewhat more by cobalt ion Lymphocytes contain very much less tripeptidase activity than polymorphonuclear leukocytes

(4) Both the glycylglycine dipeptidase and the tripeptidase are present in the soluble fraction of leukocytes

References

1 MERTEN R & M WINSCHUH 1947 L- und d Dipeptidasen in den Formelementen des menschlichen Blutes Z Vitamin , Hormon u Fermentforschung 1 351
2 FLEISHER G A 1953 Peptidases in human blood I The hydrolysis of glycylglycine and glycyl L-leucine by normal serum J Biol Chem 206 925
3 FLEISHER G A 1954 Peptidases in human blood II The hydrolysis of additional peptidases by normal serum J Biol Chem 206 637
4 BRINDLEY C O & G A FLEISHER Unpublished data
5 FLEISHER G A Unpublished data
6 FRUTON J S V A SMITH & P E DRISCOLL 1948 On the proteolytic enzymes of animal tissues VII A peptidase of calf thymus J Biol Chem 173 457
7 HOLMAN H I A WHITE & J S FRUTON 1947 Relation of adrenal cortex to serum peptidase activity Proc Soc Exptl Biol Med 65 196
8 SCHWARTZ T B & F L ENGEL 1950 Effect of adrenocorticotropic hormone and cortisone therapy on human plasma aminopeptidase activity Proc Soc Exptl Biol Med 74 82
9 CULLEN A M V T BARBER M K BIRMINGHAM & K STERN 1951 Peptidase activity in human serum under various pathological conditions in pregnancy and following the administration of ACTH Can J Med Sci 29 114
10 BUCKLEY E S JR M J POWELL & J G GIBSON II 1950 The separation of the formed elements of whole blood by means of fraction I J Lab Clin Med 36 29
11 HUSFELDT E 1931 Proteolytische Enzyme in den Leukocyten des Menschen Z physiol Chemie 194 137
12 SMITH E L 1948 Studies on dipeptidases III Hydrolysis of methylated peptides the role of cobalt in the action of glycylglycine dipeptidase J Biol Chem 176 21
13 STERN K M K BIRMINGHAM A CULLEN & R LICHER 1951 Peptidase activity in leucocytes erythrocytes and plasma of young adult and senile subjects J Clin Invest 30 84
14 AGREN G 1945 The purification and properties of aminopolypeptidase from the hog's pyloric mucosa *I Acta Physiol Scand 9 248*

A

B

C

D





Ult phot Z i Obj immers Z i 90 Oc 1
F t Z I 18 T d m 35 m Ampl 1200



F 1 O d se t ty th t ph l l g t th bl l t sc l ( ) act ty 1 (b) t ty 2 ( ) t ty 3 (d) t ty 6

and D represent the same experiment made with rabbit blood * The inactivation is obtained also by HCN which corroborates that the enzyme is oxidase

In the healthy man the oxidase is found in the eosinophils (intensity 1 or 2) and in the neutrophils (average intensity 3) but in some syphilitic patients whose treatment is beginning there are neutrophils with oxidase 5 and even 6 To have an estimate of the oxidase blood power these values must be tabulated and related to the number of leukocytes in the blood Special record cards for this purpose (FIGURE 3) were designed with 100 spaces to mark the negative oxidase leukocytes and the value of each oxidase positive Addition of the

S Appe d T h q II p g 1047

OXIDASE AND LIPASE OF THE LEUKOCYTE

Research Work that Resulted in a Chemical Blood Test to Detect Infections by Acid Fast Bacilli

By Paulo Seabra

Academia Nacional de Medicina Rio de Janeiro Brasil

As emphasized by Doctor Doan *et al* in his paper "Maturation of Myelo blasts' the oxidase content of the neutrophil shows the degree of its matura tion[1] but at the same time, Fiessinger, in his very good book on leukocytes concluded with these words the method which would permit a judgment on the functional conditions of the leukocyte is still to be discovered

I was endeavoring to find such a method when Loele published his process of stable oxidase staining of myeloid leukocytes[3]

The blood smear is fixed with formaldehyde vapor covered with a "mordant solution containing a naphthol and is oxidized giving a pale brown color with an intensity proportional to the oxidase activity The smear is then washed with water and covered with a gentian violet solution A naphthol adsorbs the gentian violet and becomes stained a blue color The final result is that the blue color is observed where the oxidative reaction took place, and the intensity of the blue color in cells is proportional to the activity of the oxidase in that cell or granulation * I tried this method with thoroughness but in spite of the wonderful stability of the blue stain its intensity was very variable in smears of the same blood

Loele uses alcohol in his staining solutions and I verified the fact that this substance activates or inhibits the oxidase action according to the stage reached in the drying process of the blood smear For this reason aqueous solutions were substituted for alcoholic solutions and satisfactory regularity was obtained A color scale going from white to blue in six steps was used as a standard to meas ure the color intensity FIGURE 1 reproduces this scale and gives an example of four neutrophils A' showing intensity 1 B' intensity 2 'C intensity 3 and D intensity 6

I do not refer to the quantity of oxidase used but only to its activity because as emphasized by Ammon[4] in regard to enzymes we do not know whether the variations in activity are caused by variation in the amounts of the enzymes present or by variation in the amount of the elements that activate or inhibit *their action*

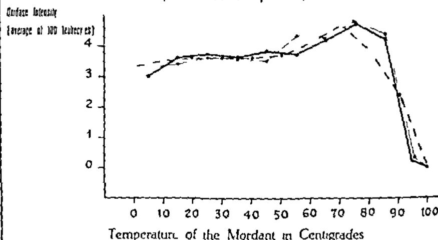
The enzymatic character of the staining process is demonstrated by heating GRAPH 1 shows the intensity of the blue color and consequently the oxidase ac tivity as a function of the temperature We can see that as the temperature goes up there is a progressive activation of the enzyme up to 75° C followed by a sudden fall at 80 C arriving at O at 100° C

In FIGURE 2 A is a neutrophil of an apparently healthy man B is from the same blood but the smear was heated at 100° C for 10 minutes C

* See Append Techq I pag 104

Man	Man	R t
Apparently healthy	Syphilitic	
D t N 3 1937	Date Oct 11 1937	Date-May 7 1938
Obs. S. S.	Ob T Mov 1 F A M.	Obs. Rat № 59
122 5 / 246 0	168 0 / 336 0	39 0 / 75 5
O in 00 L / L mm3	Ox. in 00 L / L p. mm3	Ox. in 00 L / L p. 3
66 11	174 00	165 00
1476 / 1477 / 1623	2337 4 / 36 / 846	375 / 458 / 23 / 723 75
OXIDASE INDEX 16 2	OXIDASE INDEX 58 5	OXIDASE INDEX 12 4
Observ	Observation	O rv

F u 3 Sp l lf d g d l l t gth d se d



GR P I 1 D mo tr t o f th e zym t h te of the sta g p oce s

A

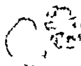


B

C



D



F g 2 D m t t f th zym t h t f th lase (ed b j h t g t I (f th m r (b) nd ( ) ea) I se j m t t lymphocyt

Man Apparently healthy D t Nov 3 1937		Man Syphilitic D te Oct. 11 1937		R t D t May 7 1938	
Obs. S. S.	122 5	Obs. 7 Man	F A M	Obs. Rat № 39	24 8
122 5	246 0	768 0	336 0	39 0	25 5
O in 001 L p. mm3	66 22	Ox in 001 L p. mm	774 00	O in 1001 L p. mm3	165 00
	16236		3846		723 75

OX ASE I DEX 16 2 O ASE I DE 58 5 OXIDAS DE 12 4

Observation Observation D care

F 3 Spec 1 d f 1 g 1 lcul t g th lase nd

values found in 100 leukocytes expresses the leukocytic oxidase By multiplying this number by the hundreds of leukocytes in each cubic millimeter and keeping only the first decimal figure as shown in FIGURE 3, the oxidase index is obtained

FIGURE 3 shows the oxidase index, 16 2, of a healthy man, the index 58 3 of a syphilitic man, and the index 12 4 of a rat The average for a normal man is 15 2

I introduced the oxidase index in 1940 and many important papers based on it were published in several countries[5-14] but I recognize that personal factors in evaluating the blue color of each leukocyte reduces its accuracy

Going now to another stage of this work all the stained smears have a pale blue aspect whose intensity I observed, varies from one person to another This staining presents the same enzymatic characteristics as that of the neutrophils, but it was rather difficult to locate the enzyme in the plasma or in the erythrocytes because both appear almost unstained under the microscope Meanwhile if in the same microscopic field we examine an untreated region of the smear a region treated only by the mordant, another treated only by gentian violet and finally another treated by both only in this last portion can we

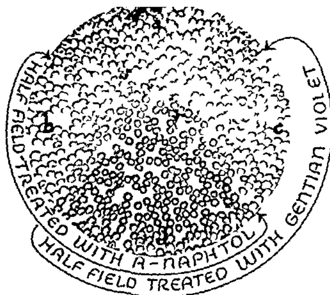
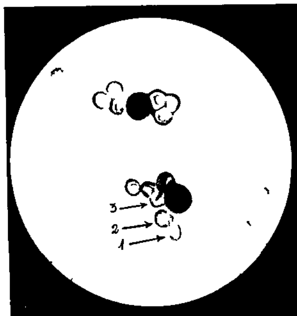


FIGURE 4 I u t t on of th eyth ocyt o d se I th sam m oscop fi ld a be e [illegible] t at d rythrocytes (b) erythrocytes t ted w th n phth l ( ) treated w th g tia [illegible] l t (d) t ted th both a-n phth l d g nt an iolet These a the ly e yth ocytes t becom [illegible] ed



F g 5 Ill t t g hype ff Th d se i th k d l q d l es th t ph l d l ps those ryth ocytes los p m ty Th d g f t ty f h p ff ted 1 2 3

see the blue color and this color is not in the interglobular spaces but in the erythrocytes (FIGURE 4) *

Therefore the mammalian erythrocyte has some oxidase although the texts do not mention it It appears to me that this small amount of enzyme comes from the neutrophils by physiological effusion

This hypothesis was suggested to me by the fact that it is very common to find in the blood smears close to perfect neutrophils others in which the oxidase effuses as a viscous liquid and agglutinates the erythrocytes nearby There is therefore a variation in the stability of the neutrophils and the hyper effusion is a sign of instability

To find an approximate measure of the stability of the neutrophils in a blood sample a hypereffusion test was conceived The intensity of coloration of the erythrocytes close to each neutrophil is evaluated and expressed in degrees 1 2 or 3 as shown in FIGURE 5 and the total reading of the smear is expressed as (−) (+) (++) (+++) or (++++) **

Tabulating the results of the hypereffusion test made on blood of 50 industrial workers (CHART 1) it was found that there is a marked difference in the stability of the neutrophils in some people as compared with others Looking for some endocrine influence CHART 2 was obtained suggesting that hyper

S App d T h q III p g 1047
S App d T h q IV pag 1047

CHART 1
HYPEREFFUSION TEST
Fifty apparently healthy workers

Date (1942)	No	Name	Results				Date (1942)	No	Name	Results			
October 1	1	P S				±	October 20	28	J M				+
5	2	H K				−		29	B M			+	+
	3	L M				+		30	M P				−
8	4	L C				±		31	B S			+	+
	5	D S				±		32	A S				+
	6	M R G				±	21	33	N A				−
	7	J V				−		34	N F				−
	8	H P				+		35	I M				+
	9	C S				−		36	A C				±
10	10	C B				+		3[illegible]	L B				−
	11	V P				−		38	M C			+	+
	12	N V				+		39	D B				−
	13	I O				−		40	S S			+	+
14	14	A C				−	2[illegible]	41	I N				−
	15	H B				±		42	E M		+	+	+
	16	A P				+		43	I P				−
	17	M A				±		44	F S	+	+	+	+
	18	V M V				−		45	F P		+	+	+
15	19	W I				+		46	P F M			+	+
	20	A N L			+	+		47	I S		+	+	+
	21	I I				−		48	O C				+
	22	I S			+	+	29	49	L C				+
17	23	A V			+	+		50	V F		+	+	+
	24	I M S				+							
	25	O S		+	+	+							
	26	A C			+	+							
	27	L C			+	+							

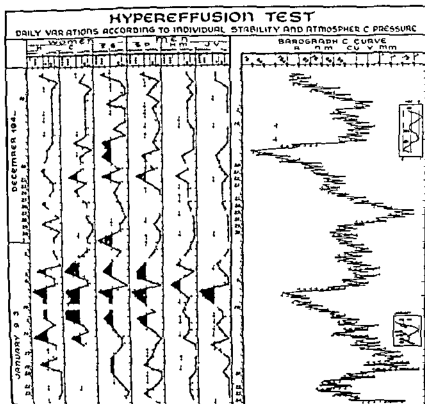
Results appearing only on the first column (− ± and +) 68%
Results appearing on the other columns also (++ +++ and ++++) 32%

The Hypereffusion Test shows that there are persons whose neutrophils are more stable than others On some days hypereffusion is observed to be more intense than on others

CHART 2
LEUKOCYTIC OXIDASE IN CASES WITH HYPOTHYREOIDISM AND HYPERTHYREOSIS
(Patients from the Department of Endocrinology General Polyclinic Rio de Janeiro Brazil)

Hyperthyreosis			Hypothyreoidism		
Name	Oxid.	Total leukocyt	Name	Oxid.	Total leukocyt
		Ratio			Ratio
S F	186.0		D M	248.5	
C S B	104.5		N N C	24[illegible].0	
F B	188.0		F A G	217.5	
B B B	229.5		D I	193.0	
S C	166.0				
I V N	18[illegible].0	176.8			22[illegible].2

Hyperthyreosis patients presenting low leukocytic oxidase due to high leukocytic instability



Graph 2 Demonstration that the hypereffusion [illegible] most raised during periods of atmospheric depression

thyreosis contributes to hypereffusion with leukocytic oxidase average 176 when the hypothyreosis averaged 225

CHART 1 also shows that there is a noticeable variation in the intensity of the hypereffusion from day to day Many factors were examined in the search for an explanation of this variation It was finally observed that the barometric pressure was the factor involved GRAPH 2 shows the hypereffusion curve of six persons and the variation in barometric pressure for the same period It can be observed that the two waves of high hypereffusion correspond to the two strong drops in the atmospheric pressure

Considering that intense atmospheric depressions induce agitation in the mentally unstable it should be interesting to check up any relationship between neutrophilic hypereffusion and mental agitation in an institution for the mentally ill Blood was taken at the same moment from patients under agitation and from patients while calm The results in CHART 3 show an intense hypereffusion in the patients under agitation This finding is mentioned here only as a stimulus to further work in this field

CHART 3
HYPEREFFUSION TEST
(Mentally unstable patients during periods of calm and agitation)

Period of	Patient	Diagnosis	Results			
			++	+	±	−
Calm	A	Alcoholism and epilepsy				x
						x
						x
	M C	Paranoid schizophrenia			x	
					x	
						x
	A	Manic depressive psychosis				x
						x
					x	
	F O	Autotoxic psychosis				x
					x	
						x
Agitation	L	Mental debility and schizophrenic reaction			x	
					x	
					x	
	M C	Manic depressive psychosis			x	
			x			
			x			
	F P	Schizophrenia	x			
					x	
	M B	Alcoholism syphilis		x		
				x		

The mentally unstable in agitation periods shows high neutrophil instability

An effort was made to understand the atmospheric pressure influence on the oxidase effusion The first step was to observe that the oxidase shift from the white to the red cells is not a consequence of a uniform dissolution of the enzyme for in many smears close to the stained erythrocytes we can see the neutrophil with empty spaces of one or more granulations that attracted the enzyme FIGURE 6 is an example of this

My hypothesis was that the neutrophil granulations are formed by a surrounding membrane containing the enzymatic viscous liquid

Rebuck's splendid electron micrographs (FIGURE 7) bring strong support to this hypothesis as he himself emphasized because the granulations appear more transparent in the center than at the periphery contrary to the way they would appear if they were homogenous solids[15]

If the surrounding membrane has a progressive maturation process we can understand that in each neutrophil only the granulations that are already ripe do not resist atmospheric decompression and burst In similar fashion the ripe membrane does not resist the contraction produced by the formol vapor which is used to fix the smear in the staining process When covering the slide with the liquid mordant the enzyme of the ruptured granulation is diluted and spreads as shown in FIGURES 5 and 6

The membrane of the broken and empty granulations free from any oxidase

F g 6 N t ll eut ph l ffected by hype eff n Th t f rm d ssol t on patt rn f th enzym f lose pro m ty t th rst ed yth xcytes ( ) w oc a n lly see empty pac left th eutroph l by th t f rred enzym (b)

Fi u 7 R b k l t m g ph how th t th t oph l gr n l t s les dense in th te th th per ph ry wh h ppo t th th hypoth th t these g ul t f rmed by a fl d conta ned by memb a

CHART 3
HYPEREFFUSION TEST
(Mentally unstable patients during periods of calm and agitation)

Period	Patients	Diagnosis	Result			
			++	+	±	−
Calm	A	Alcoholism and epilepsy				x x x
	M L	Paranoid schizophrenia			x x	
	A	Manic depressive psychosis				x x x
	F O	Autotoxic psychosis			x x	x x
Agitation	L	Mental debility and schizophrenic reaction			x x x	
	M C	Manic depressive psychosis	x x		x	
	F P	Schizophrenia	x		x	
	M B	Alcoholism syphilis		x x		

The mentally unstable in agitation periods shows high neutrophil instability

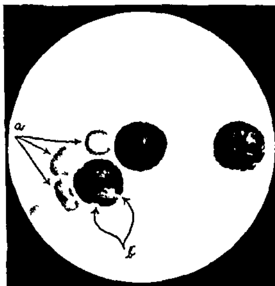
An effort was made to understand the atmospheric pressure influence on the oxidase effusion The first step was to observe that the oxidase shift from the white to the red cells is not a consequence of a uniform dissolution of the enzyme for in many smears close to the stained erythrocytes we can see the neutrophil with empty spaces of one or more granulations that attracted the enzyme FIGURE 6 is an example of this

My hypothesis was that the neutrophil granulations are formed by a surrounding membrane containing the enzymatic viscous liquid

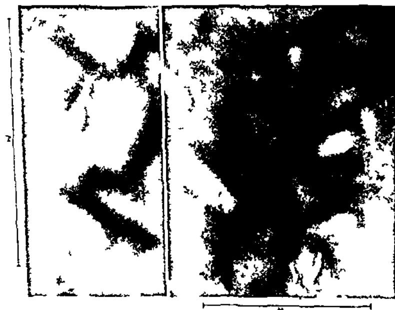
Rebuck s splendid electron micrographs (FIGURE 7) bring strong support to this hypothesis as he himself emphasized because the granulations appear more transparent in the center than at the periphery contrary to the way they would appear if they were homogenous solids[15]

If the surrounding membrane has a progressive maturation process we can understand that in each neutrophil only the granulations that are already ripe do not resist atmospheric decompression and burst In similar fashion the ripe membrane does not resist the contraction produced by the formol vapor which is used to fix the smear in the staining process When covering the slide with the liquid mordant the enzyme of the ruptured granulation is diluted and spreads as shown in FIGURES 5 and 6

The membrane of the broken and empty granulations free from any oxidase



F ta 6 N t ll troph ls r ffected by hype fl on Th t f m di sol tio patt f th enzym f lose j ro m ty to th t ed ry h ocytes ( ) w occasion lly see empty pa l ft th troph l by th t f red zym (b)



F u 7 R b k l t m g (h sh w th t th t (h l gr l t less d f th t th th p ph ry wh h rpo t th th hypothes that th se gr l t on a f rmed by a fl d ta ed by m mb

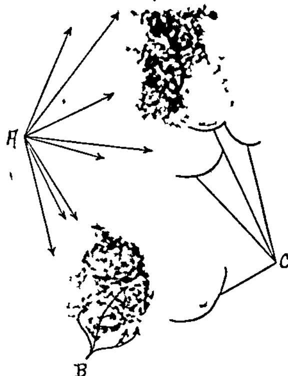


FIGURE 8 Tw ru hed t ph l ( ) gr l t sc tt ed b t t g th l se c te t (b) som mpty m mb f b t gr l t on ( ) l l q l ff sed f m b t g l t

content may be seen by crushing the neutrophils when making the smear This result is accomplished by pressing one slide strongly against the other In *A* FIGURE 8 close to two crushed neutrophils we can see scattered granulations retaining their oxidase content *B* shows the outline of some membrane from granulations that burst and therefore were not stained The oxidase effused from such membrane is found nearby as irregular spots some of which are shown in *C* Now we may understand that if an animal is exposed not to the normal atmospheric decompression but to a violent one as in a vacuum chamber there occurs a paroxysmic hyperffusion *i e* not only do the ripe granulations burst but all of them therefore the neutrophils become completely empty of oxidase FIGURE 9 shows this experiment in a monkey Before the decompression the animal s neutrophils are normal with a weak decompression (602 mm) the intense bursting of the granulations begins and when the pressure is reduced to 254 mm the neutrophils are without any oxidase at all The same thing happens to a man when he suddenly flies to a high altitude in a



F ur 9 Blood f m m k y wh h b bee bm tted t d mp Its troph ls p gr ss ly lose th 1 se

plane not equipped with pressurized cabins FIGURE 10 represents the blood of a man before and during his first flight One smear was made before the plane left the ground The neutrophils are rich in oxidase In the blood taken at an altitude of 1000 meters (3280 feet) the neutrophils appeared poor in oxidase but the erythrocytes remain almost unstained At 3500 meters (11 480 feet) the hypereffusion is paroxysmic the erythrocytes are strongly stained and the neutrophils seem completely empty of oxidase

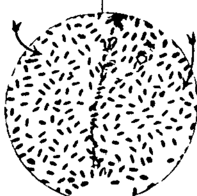
My attention was then directed to the birds the natural fliers Nothing happens in the oxidase picture of the birds Nature was wise enough the oxidase is already in the erythrocytes and not in the neutrophils as shown in FIGURE 11

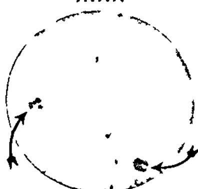
To make FIGURE 11 more striking only half the area in each of the four microscopic fields was treated with the a naphthol mordant so that we may confront in the same microscopic field cells that are stained to oxidase and those that are not stained The difference that we observe is that in man the oxidase is in the neutrophil and in birds it is in the erythrocytes A natural low altitude flying mammalian the bat (*Molossus rufus*) appears as a transition *in which the neutrophils are less stained and the erythrocytes more so as compared* with man Thanks to the Brazilian Air Force we were able to make some experimental flights GRAPH 3 represents results obtained in a fourth of these flights We can see that as the plane goes up the leukocytic oxidase goes down It is known that the blood glycolytic agent is located in the neutrophils[16,17] for this reason it seemed interesting to find if this intensive shift of the oxidase has any influence in the glycolytic function A glycemia fall was observed parallel to the fall in oxidase which uggests the importance of hypoglycemia in aviation medicine[18]



F 10 Th blood f m ft h fi t unp zed rpla fl ght h w p ysm hype ff





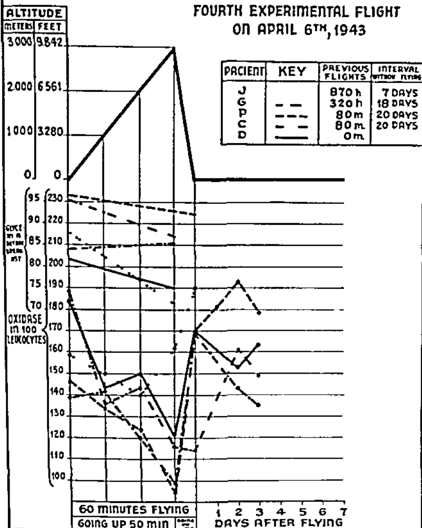




Fi t z 11 Comp g th t h lf f ld h m w se th t i the m mm l th blood d located th troph l d th t th b d t th yth oryt Th b t ppe t t st g betwee the tw

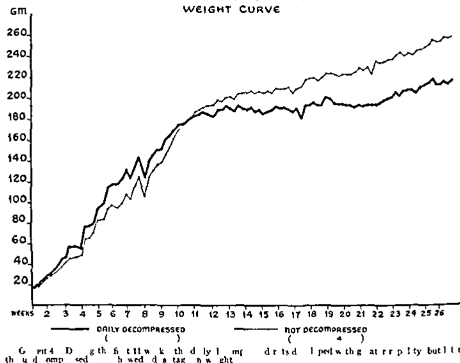
The paroxysmic hypereffusion results in an overcharge of oxidase on the erythrocytes which may be beneficial because hemoglobin needs oxidase to be oxygenated efficiently as Thomas demonstrated and when the animal is anoxemic by excess of nitrogen in the atmosphere a strong oxidase supply helps its survival [19]

As a first approach to the study of the possible advantages of this erythrocytic oxidase overcharge an experiment was made to observe the influence of decompression on the rate of growth of young rats Several lots of rats of the same breed were divided into two groups One group was decompressed daily for a few minutes in a vacuum chamber The other group was kept as control



G ra 3 As th pl goes p th leukocytic lase goes dow Th same ffect oc rs w th glycem

Graph 4 shows the growth curve for the two groups One can observe that the decompressed rats grow heavier in the first 12 weeks but that from there on their weight increases more slowly than does that of the controls By this time the neutrophils of the decompressed rats are bereft of oxidase (figure 12)



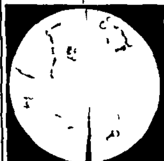
( ) (4)

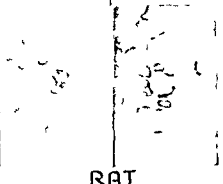
G pit 4 D g th fi t 11 w k th d ly 1 mp d r ts d l ped w th g at r r p l ty but l t r th u d omp sed h wed d a tag n w ght

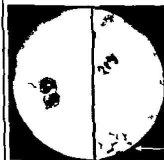
In FIGURE 12 normal rat and mouse blood are compared with blood from the same animals decompressed daily for 60 days In this same figure we compare the blood of a man who has never flown with the blood of a professional flyer taken in the morning before the latter s daily flight The blood in the latter case shows signs of adaptation presenting the oxidase picture of the bird the enzyme is in the erythrocytes and sometimes agglutinating them as in the point marked by an arrow in FIGURE 12 The neutrophils have no oxidase So what can be said when we hear from all authorities that the neutrophil defense capacity is indicated by the amount of oxidase? [9] We were able to demonstrate that rats brought to such a condition are less immune to the *Spirochaeta gallinarum* [21] a microorganism which normally infects only the birds

Going back to GRAPH 3 we see that when the plane descends and lands the average leukocytic oxidase goes up reaching the normal level for the neutrophils that were resting in the blood reservoirs such as the spleen now go into the circulation under the visceral compression produced by the plane s loss of altitude Shortly after a flight a blood smear shows many neutrophils with oxidase intensity 1 and 0 together with many with an intensity of 4 or 5 while in a blood smear taken before the flight almost all leukocytes will have presented an intensity of 3

This fact indicates that the neutrophil loses its oxidase when it leaves the reservoirs and goes into circulation This phenomenon is easy to understand







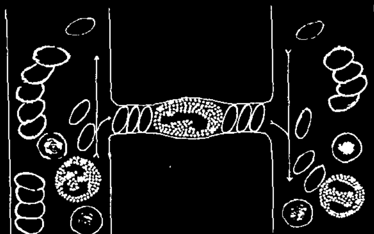


F ore 12 I m mm la d g g d ly d p es d m tak g f l fl ghts d ly th blood m l t th t f b d j se be g f j th yth ocyt t in th n t f l l S m tim th ym ppe rs ac tt ed ggl t t g th ryth ocyt show by th w

if we remember that during the blood circulation, the neutrophils suffer a physiological crushing. The diameter of the capillaries, we know, is smaller than an erythrocyte and much smaller than the diameter of a neutrophil. When we look through the microscope at the capillary circulation in the mesenterium of a rat, as shown in FIGURE 13, we can see that the neutrophil has been squeezed through the elastic capillary as represented in FIGURE 14. I believe that it is at this moment that the ripe membrane surrounding the granulation bursts



FIGURE 13. Obse[illegible]g th[illegible] [illegible]ap[illegible]ll[illegible]ry [illegible]rc[illegible]lat[illegible] th[illegible] m[illegible]se[illegible]t[illegible]m of a l[illegible]g [illegible]t



F G 14 S hem ti d gram how g how th e troph l sq zed t p sse th ugh the cap ll ry

and pours out its viscous enzymatic content to be adsorbed by the erythrocytes nearby The viscous consistency of this fluid may possibly account for the agglutination of the erythrocytes as coin stacks

But what induces the surrounding membrane to maturation? The clue for finding the ripening factor of the surrounding membrane was taken from the observation that the leukocytic oxidase level is raised in tuberculosis In the tubercular patient the granulation seems to burst less for the enzyme accumulates in the neutrophil GRAPH 5 shows the oxidase index going up as the disease advances

The same GRAPH 5 confirms many authors [25] showing that tuberculosis reduces the serum lipase activity as does leprosy [26] for the virulent acid fast bacilli produce a lipase inactivating substance named by Calmette antilecithinase and by Middlebrook Factor P [27]

Wells DeWitt and Long who mention the statement of Kollert and Frisch that lipase is connected with immunity to tuberculosis pointed out that as a matter of fact the technical difficulties are so great in the quantitative study of fat cleavage that virtually all the work reported as on lipase activity really concerns esterases splitting ethyl butyrate triacetin or tributyrin [28]

Kraut and Burger confirmed that the work with tributyrin only confused the lipase study in tuberculosis By utilizing as substrate a true fat extracted from the bacillus they concluded that the blood hydrolyzes this fat not by the pancreatic lipase but only by the leukocytic lipase [29]

Barnes working carefully with cats confirmed more recently the claims of

if we remember that during the blood circulation the neutrophils suffer a physiological crushing The diameter of the capillaries we know, is smaller than an erythrocyte and much smaller than the diameter of a neutrophil When we look through the microscope at the capillary circulation in the mesenterium of a rat as shown in FIGURE 13 we can see that the neutrophil has been squeezed through the elastic capillary as represented in FIGURE 14 I believe that it is at this moment that the ripe membrane surrounding the granulation bursts



FIGURE 13 Observing the capillary circulation in the mesenterium of a rat



Fig 15 O ly th t ph l gra l t bec m t ed blood m t ted by th Z hl Neelse modified m thod

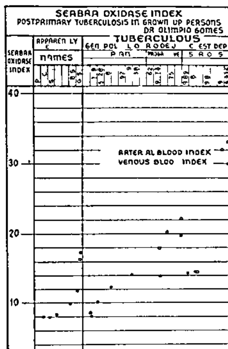
This physiologic correlation is upset by the virulent acid fast infections that inhibit the lipase activity accumulating the oxidase in the neutrophils and reducing its presence in the erythrocytes From this activity a diagnostic possibility may be visualized

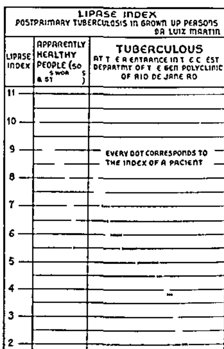
As the oxidase index is wearisome and inaccurate an electrophotometric method was developed to estimate the erythrocytic oxidase *

This method confirmed the lowering of the erythrocytic oxidase in tuberculosis and leprosy[37] but was not satisfactory for survey purposes because the general level of the erythrocytic oxidase oscillates according to the daily atmospheric pressure as shown in GRAPH 6 [33]

In 1948 at thi disappointing stage I received a letter from Professor Downey in which he made the stimulating remark that a blood test for tuberculosis

* S Appe dix Tech q VI pag 1048





G 5 T be culos d b gh d se d x a d low l j i

Fontes Bergel and Fiessinger that the lymphocyte is the leukocyte that produces lipase [30]

Knowing this fact it is possible to assume that if the surrounding membrane of neutrophil granulation has a lipidic nature as has the surrounding membrane of the acid fast bacilli the lipase produced by the lymphocytes may hydrolyze the granulation membranes that are in the neutrophil periphery ripening it

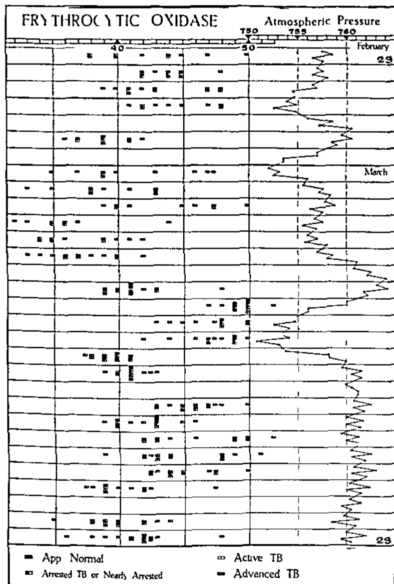
But to accept this hypothesis it would be necessary to prove first that the surrounding membrane of the neutrophil granulation is acid fast because as Lamann says the acid fastness is concerned with the lipidic membrane This proof was rendered possible through a simple adaptation of the Ziehl Neelsen staining procedure *

Staining a slide half covered by the sputum smear of a tuberculous patient and half by a blood smear we find that only the bacilli and the neutrophil granulation take the stain

FIGURE 15 is a microphotograph of blood stained by the Ziehl modified method [31]

Summing up all this knowledge we must recognize the fact that there is a functional correlation in the blood between different types of cells the neutrophils supply the erythrocytes with oxidase but to do so they need lymphocytic lipase as represented in FIGURE 16

* See th Appe dix Tech q \ pag 104



G 6 Eryth ocyt t l w t b cul pat ts b t ea ll p rso d g pe od f
tmosph d p

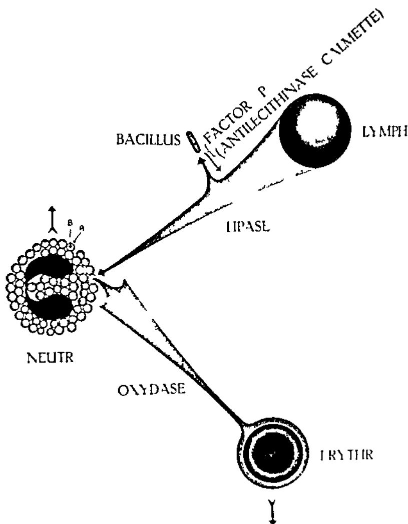


FIGURE 16 S h m t p se t t f th p posed f t l l t b tw th blood l m t d th eff t wh h f t f m d f t b ll h th l t

would be greatly welcomed by all phthisiologists I returned to the lipase work in an attempt to solve the mentioned technical difficulties in assaying it in the blood Using the Cherry and Crandall method [24] a reagent was developed This reagent is the oil substrate stabilized to permit storage and standardization * This reagent permitted the lipase assay in the routine of the clinical laboratory with an error limit of 3 per cent at the normal level

* See Appendix Technique VII page 1049

As a counterpart to this case an old arrested tuberculosis case appeared with a large pleural effusion but as the lipase titer was 8 0 the treatment used was not streptomycin but penicillin Recovery was prompt [43]

Recently Doctor Alvaro Dias in charge of the Rio de Janeiro Public Health Service opened an Experimental Lipase Survey Post and I shall be happy to close this communication by presenting for the first time the results of the first 500 tests performed there with my reagent and simultaneously controlled by photofluorography in applicants for health certificates (GRAPH 7) [41]

Attention must be paid to the fact that the individuals examined in this test thought themselves healthy Of these unemployed persons 483 showed a lipase titer of 6 5 to 9 5 Only 17 presented lipase titer of 6 0 or less

The tubercular diagrams of every patient made by the photofluorographic method corresponded to the findings made by the lipase titer in the lower group The Institute of Leprology has just reported to me that the reagent places all the 21 leprosy cases examined in this same group [42]

May I conclude by telling in a few word the story of a patient whose radiologic diagnosis was made by a full size roentgenogram? A young mother owing to a negative photofluorography obtained a health certificate She accordingly got a job to support her baby as her husband had left her Because the lipase titer in her case was only 4 the case was opened for re-examination A full size roentogenogram confirmed tuberculosis The phthisiologist explained to her that now a blood test gave more sensitive findings than photofluorography She regretted the discovery of such a test By chance I was present I told her that her disease had been discovered so early that it could be arrested in a short time She was not convinced and started to cry and cough The sputum was examined at once and revealed an average four bacilli in each field I told her that perhaps the new blood test had made it possible to prevent her from infecting her own baby Then she smiled

Appendix

Technique I

The Loele Seabra Method (name given by the National Academy of Medicine Rio de Janeiro) The blood smear must be thin and should not reach the borders of the slide Dry the smear in the open air for about two hours and then fix it by exposing it to formol vapors for 10 minutes This exposure is effected by placing the slide vertically in a vessel containing an evaporating dish with 20 cc of formalin dissolved in 40 cc of distilled water In the cold season this exposure must be made in an incubator at 37 C

Cover the smear with mordant for 15 minutes and then wash it gently with distilled water for about one minute Cover the smear with aqueous gentian violet for 10 minutes and wash under a fine stream of distilled water for five minutes Cover the smear with aqueous basic fuchsin solution for 10 seconds and wash under a fine stream of distilled water for five minutes After each washing tilt the slide vertically and let it stand until it is completely dry

Solutions Gentian violet dissolve 0 1 gm in 250 cc of distilled water and

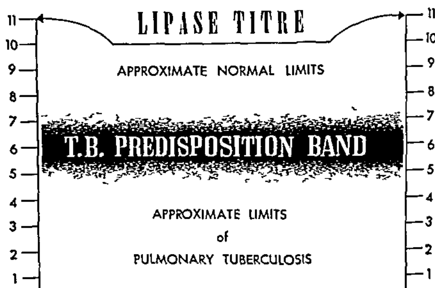


FIGURE 17. Lipase scale.

The lipase titer is not dependent on personal interpretations as tuberculin or photofluorography readings are for it is obtained by a simple titration with sodium hydroxide.

The significance of the lipase titer is resumed in a lipase scale (FIGURE 17) presented in a paper on "The Social Importance of the Lipase Survey."[35] In this paper the Ryang experimental conclusion that the tuberculosis-predisposed animals are poor in lipase[36] is quoted and so are the Mayer and Rappaport statements that "the transition from prephthisical to phthisical status may take many years" and "it is a logical assumption that treatment of the prephthisical lesion may prevent development of phthisis."[37]

A thesis was presented at the University of Mexico on the lipase titer in tuberculosis using the reagent which I developed.[38] The reagent is also being used by a few phthisiologists in France and by many in my country. These clinicians are finding very close agreement between the lipase test and the clinical and radiological picture. Some disagreement verified in cases studied by the Tuberculosis Service of the Brazilian Air Force deserves special attention: two patients whose radiologic and clinical finding simulated tuberculosis presented a high lipase titer, *i.e.* 7 and 7.5. The tuberculosis treatment was ineffective and autopsy revealed pulmonary schistosomiasis in the one and pulmonary sarcoidosis in the other. An old case of arrested tuberculosis came to the Tuberculosis Service presenting very bad clinical conditions. The lipase titer was 2.0 although the chest roentgenogram showed no sign that the old pulmonary lesions had regained activity. A sudden death by an intestinal rupture occurred and the autopsy revealed extensive tuberculosis ulcerations. In another case tuberculin and bacteriologic findings were negative despite the advanced pulmonary involvement. As the lipase titer was 3.0 the tuberculosis treatment was begun and proved to be correct by its gratifying results.[39]

decant on the following day Basic fuchsin dissolve 0 1 gm in 150 cc of cold distilled water decanting on the following day Mordant this preparation does not keep more than one day It must be stored as two separate solutions and mixed each time to form the desired volume Solution A —dissolve 0 2 gm of a naphthol in 350 cc of distilled water and filter solution B —dissolve 0 8 gm of ammonium carbonate in 50 cc of distilled water Mix in the proportion of 3 0 of A to 50 of B boil for five minutes and allow to cool Restore to initial volume by adding cold water Place in the incubator at 37 0° C for two hours before using

Note The handling of the slides while washing requires the greatest care and attention especially during the hot season for as the smears are not fixed in alcohol nor colored with alcoholic stains but are exposed to alkaline mordant and basic staining they are likely to puff up forming folds and to stand up from the slide thus being easily carried away with the water

Technique II

The heating of blood smears at 100° C (FIGURE 18) Two slides (a) are joined face to face with the blood smears in the inner side A cartoon joint (b) prevents the smears from touching each other Two rubber strips (c) hold the slides together

The two slides are placed in a beaker under another beaker half full of mercury Mercury is then poured between the two beakers completely covering the slides The whole assembly is placed into a larger beaker containing a layer of glass beads and is half filled with water

Technique III

One microscopic field showing erythrocytes in the various stages of the staining process The Loele Seabra method is used but the smear is not completely covered with the mordant solution Only one drop is used After washing one drop of the gentian violet is used lateral to the mordant drop so only a little portion of smear receives the mordant and the violet gentian The microscope is focused where the limbs of the two drops cross FIGURE 19 is self explanatory

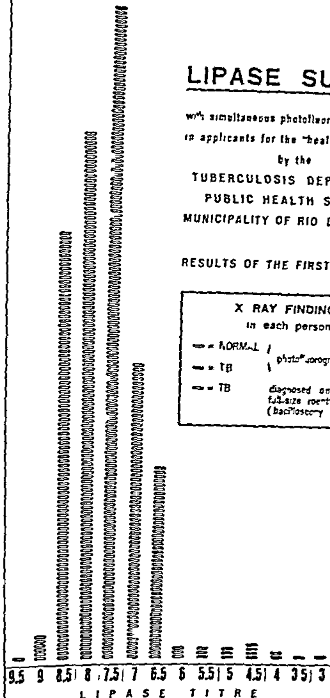
Technique IV

Hypereffusion test Examine four distant microscopic fields containing granulocytes in the center of the smear and also four distant fields on the borders on both borders if possible Observe the most intense points in each field using the scale shown in FIGURE 5

Results ++++ when intensity 3 is found in the eight fields +++ when intensity 3 is found in the center ++ when intensity 3 appears only in the borders of the smear + when only intensity 2 is found ± when only intensity 1 is found and — if no hypereffusion is found

Technique V

Ziehl modified staining process (1) Dry the smear in open air for two hours (2) fix in formol vapors (20 cc formalin dissolved in 40 cc of distilled water)



Graph The beginning of the first official lipase survey

decant on the following day Basic fuchsin dissolve 0 1 gm in 150 cc of cold distilled water decanting on the following day Mordant this preparation does not keep more than one day It must be stored as two separate solutions and mixed each time to form the desired volume Solution 'A —dissolve 0 2 gm of a naphthol in 350 cc of distilled water and filter, solution B —dissolve 0 8 gm of ammonium carbonate in 50 cc of distilled water Mix in the proportion of 350 of A to 50 of B boil for five minutes and allow to cool Restore to initial volume by adding cold water Place in the incubator at 37 0 C for two hours before using

Note The handling of the slides while washing requires the greatest care and attention especially during the hot season for as the smears are not fixed in alcohol nor colored with alcoholic stains but are exposed to alkaline mordant and basic staining they are likely to puff up, forming folds and to stand up from the slide, thus being easily carried away with the water

Technique II

The heating of blood smears at 100 C (FIGURE 18) Two slides (a) are joined face to face with the blood smears in the inner side A cartoon joint (b) prevents the smears from touching each other Two rubber strips (c) hold the slides together

The two slides are placed in a beaker under another beaker half full of mercury Mercury is then poured between the two beakers completely covering the slides The whole assembly is placed into a larger beaker containing a layer of glass beads and is half filled with water

Technique III

One microscopic field showing erythrocytes in the various stages of the staining process The Loele Seabra method is used but the smear is not completely covered with the mordant solution Only one drop is used After washing one drop of the gentian violet is used lateral to the mordant drop so only a little portion of smear receives the mordant and the violet gentian The microscope is focused where the limbs of the two drops cross FIGURE 19 is self explanatory

Technique IV

Hypereffusion test Examine four distant microscopic fields containing granulocytes in the center of the smear and also four distant fields on the borders on both borders if possible Observe the most intense points in each field using the scale shown in FIGURE 5

Results ++++ when intensity 3 is found in the eight fields +++ when *intensity 3 is found in the center* ++ *when intensity 3 appears only in the* borders of the smear + when only intensity 2 is found ± when only intensity 1 is found and — if no hypereffusion is found

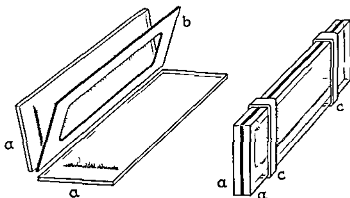
Technique V

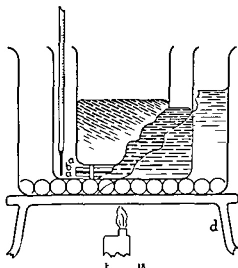
Ziehl modified staining process (1) Dry the smear in open air for two hours (2) fix in formol vapors (20 cc formalin dissolved in 40 cc of distilled water)

for four minutes placing the vessel first in the incubator at 37 C for at least half an hour (3) cover smears with Ziehl fuchsin let stand for 15 hours (4) rinse with distilled water until the water shows no color (5) discolor the smear with pure alcohol by adding 0 5 per cent of nitric acid (d = 1 40) When using this process the smears must be thin

Technique VI

The erythrocytic oxidase estimation The author considers that his photoelectric process still needs further development The process was fully described in *Diseases of the Chest* Vol XII (6) 1946 p 552





F 18

Technique VII

The Lipase Reagent Preparation oil emulsion Formula neutral cotton seed oil (maximum acidity $^{1}/_{4}$ per cent 100 ml acacia U S P —5 gm sodium carbonate anhydrous (sufficient to neutralize gum) distilled water q s p 100 ml

Preparation Take as many cubic centimeters of a 1 per cent anhydrous sodium carbonate solution as necessary according to a previous test to neutralize 5 gm of acacia taken from the same batch and complete 100 ml with distilled water dissolving the acacia in this liquid

This neutralized acacia solution is placed in a mixer (blender) and the oil is added drop by drop

Homogenization will take place after 15 minutes Then the solution is placed in a vacuum chamber until gas production has ceased

Solution is distributed in ampules of 5 ml immediately and sterilized at 120 C for 20 minutes on the same day of its preparation

Buffer Solution Formula potassium phosphate monobasic 8 31 grams sodium phosphate dibasic 5 52 grams distilled water q s p 100 ml

Preparation dissolve salts in distilled water Filter distribute in ampules of 5 ml and sterilize at 120 C for 20 minutes

Use (1) Into a wide mouth test tube or into a small Erlenmeyer put 1 ml of the patient s serum add 2 ml of the oil emulsion 3 ml of distilled water and 0 5 ml of the buffer solution Shake and place in an incubator at 98 6 F (37 C) for 24 hours

(2) Into another receptacle like the one previously described prepare a control by using 4 ml of distilled water 2 ml of oil emulsion and 0 5 ml of buffer solution Shake and place in the same incubator for 24 hours

(3) After 24 hours add 3 ml of neutral ethyl alcohol to each tube Titrate

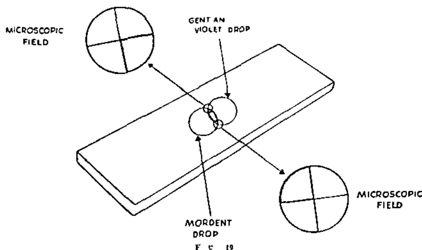


Fig 19

with N/20 sodium hydroxide and one drop of 1 per cent phenolphthalein solu tion until a rose color appears and is maintained for at least one minute

Warning (1) The blood must be withdrawn while the patient is fasting

(2) The serum must be obtained by natural coagulation and must be slightly centrifuged after retraction of the coagulum

(3) Titration time is at the minimum 24 hours after placing the tubes in the incubator and 26 at the maximum if accurate results are to be obtained

(4) As is known the N/20 sodium hydroxide should be kept in small flasks completely filled so as to avoid alteration by the atmospheric CO or should be protected by some device

(5) Since the volume used is small it is advisable to use microburettes

(6) Make sure that soap or other residues do not pollute the receptacles

Evaluation The difference between the number of cubic centimeters of sodium hydroxide solution used in the first and second tubes determines the lipasic activity of the blood serum The lipase titer is that difference multi plied by ten

Example In the first tube 1 8 ml of N/20 sodium hydroxide were used and in the second 1 0 ml The difference is 0 8 and the lipase titer is 8 0

Oil Emulsion Seabra s Lipase Reagent is packaged in cartons containing 60 ampules of 5 ml of the oil emulsion (ampules in white wrapper) Before opening the ampule its contents must be completely homogenized by adequate shaking For each determination 4 ml are used When making several de terminations at the same time the remainder of the material may be collected and used The excess should be discarded however and must not be kept for later use

Buffer Solution Every box of Seabra s Lipase Reagent contains one ampule containing more than 5 ml of the buffer solution (ampule in green wrapper) Five ml measured exactly are dissolved in 70 ml of distilled water The solution is kept in a flask ready for use at any time and is sufficient for 75 de terminations

References

1 Sabin F R C R Austrian R S Cunningham & C A Doan 1942 Studies in the maturation of myeloblasts into myelocytes and on amitotic cell division in the pe ripheral blood in subacute myeloblastic leucemia J Exptl Med **40** 845

2 Fiessinger N 1923 Les ferments des leucocytes en physiologie et thérapeutique générale Masson Paris France

3 Loele W 1936 Eine Dauerfarbung der Oxydasen in myeloidischen Leukozyten im Blutausstrich Deut med Wochschr **62** 2004

4 Ammon R 1936 Die Fermente unter pathologischen Bedingungen Deut med Wochschr **62** 1958

5 Seabra P 1939 Índice de oxidase Bol acad nacl med **3** 9

6 Pessanha J J & W Lins Filho 1942 Índice de oxidase Seabra no diabetes mellitus Imprensa Médica **19** 360

7 Coelho O & W Lins Filho 1943 Índice de oxidase Seabra na fisiologia do vôo de altitude Imprensa Médica **19** 360

8 Echave D 1944 Relação entre o índice de oxidase Seabra e a cupremia normal e patológica Imprensa Médica **20** 362

9 Lins Filho W & M de Magalhaes Prado 1945 Índice de oxidase Seabra no tra balho mental O Hospital **27** 4

10 Souto Maior M C 1946 Oxidase and diabetes Medical Times **7** 74

11 LINS FILHO W & J J PESSANHA 1946 Índice de oxidase Seabra no hipertiroidismo Medicina Cirurgia e Farmácia 120 (April)
12 GOMES O 1942 Índice de oxidase na tuberculose pulmonar post primária no adulto Rev brasil tuberculose 11 84
13 PESSANHA J J & W LINS FILHO 1947 Oxidase e cancer Imprensa Médica 23 404
14 LINS FILHO W & P DE SIQUEIRA DELDUQUE 1947 Oxidase e manipulação crônica de gasolina de aviação A Patologia Geral 32 11
15 REBUCK J W 1950 Ultrastructure of leukocytic granules and their electron microscopy Mich Acad Sci Arts & Letters Vol 36 (Published 1952)
16 SOFFER L J & M M WINTROBE 1932 The metabolism of leukocytes from normal and leukemic blood J Clin Invest 11 661
17 VIGNOLI J 1938 Fisiologia da Regulação Glicêmica Rio de Janeiro Brazil
18 SEABRA P 1946 Oxidase and aviation Medical Times (June)
19 THOMAS J A 1953 Nouveaux aspects du problème de l'hématose le facteur enzymatique de fixation de l'oxygène sur l'hémoglobine La Pathologie Générale No 644 90–100 1953 Propriétés de la globine oxydase et son action oxygénante chez l'animal Compt rend 236 415–417 1952 L enzyme catalysant l'oxygénation de l'hémoglobine Compt rend 235 1537 1539 1952 Le rôle de facteurs enzymatiques extraits de poumon ou du coeur sur l'oxygénation de l'hémoglobine Compt rend 235 318 320
20 SCHLECHT H Doenças do sangue Portuguese translation from the German by R Margarido São Paulo Brazil
21 SEABRA P 1952 Oxydase und Immunität Z Hyg 135 499–504
22 PORTER A F 1916 The distribution of esterases in the animal body Biochem J 10 523
23 WOLF J E 1939 Vitamin and ferment contents of blood in course of infectious disease in high mountains J Am Med Assoc 112 677
24 COSTANTINI G & G TOSI 1939 Lipasic power in pulmonary tuberculosis J Am Med Assoc 110 1,96
25 MARTINS L S 1934 Do índice de lipase no Rio de Janeiro e suas variações na tuberculose pulmonar Rev brasil tuber (Year III No 10) 1,3
26 ROGERS L 1923 Note on Boelke s method of using sodium morrhuate in tuberculosis Brit Med J 2(1249–58) 1253
27 MIDDLEBROOK G 1945 Pathogenic components of the tubercle bacillus II Enzymes in tuberculosis Am Rev Tuberc 61 260
28 WELLS H G L M DEWITT & F R LONG 1923 The Chemistry of Tuberculosis Williams & Wilkins Baltimore Md
29 KRAUT H & H BURGER 1938 Die Spaltung von Tuberkelbazillfetten durch Lipase Z Physiol Chem 253 105–122
30 BARNES J M 1940 The enzymes of lymphocytes and polymorphonuclear leukocytes Brit J Exptl Path 21 264–275
31 SEABRA P 1951 The surrounding membrane of the granulations of the neutrophil and its acid alcohol fastness Blood 6 (10)
32 SEABRA P 1946 Oxidase and tuberculosis Physiopathologic meaning and possibilities for the diagnosis of infections by acid fast bacilli Diseases of the Chest 12 (6)
33 SEABRA P 1948 Corrélation Physiologique entre les globules blancs et rouges du sang Bull acad suisse sci médicales 4 391–414
34 CHERRY I S & L A CRANDALL JR 1932 The specificity of pancreatic lipase its appearance in the blood after pancreatic injury Am J Physiol 100 266
35 SEABRA P 1954 Die Soziale Bedeutung des Lipasekatesters Z Hyg 139 121 129
36 RYANG C 1933 Über einige Beziehungen zwischen Lipase und Tuberkulose Thesis Freiburg i Br
37 MAYER E & I RAPPAPORT 1945 Prephthisic tuberculosis J Am Med Assoc 127 16
38 HAYASHI BECERRA S 1953 Indice de lipasa y tuberculosis Thesis Mexico City D F Mexico
39 D ANDRETTA J C Personal communication regarding results obtained by Brazilian Air Force
40 LOMBA P Personal communication regarding results in his private office
41 FERNANDES R W MOREIRA DA CUNHA & O CULLINER VICENTE Personal communication regarding the municipal lipase survey
42 RISI J B Personal communication regarding results obtained by the Instituto de Leprologia

with N/20 sodium hydroxide and one drop of 1 per cent phenolphthalein solution until a rose color appears and is maintained for at least one minute

Warning (1) The blood must be withdrawn while the patient is fasting

(2) The serum must be obtained by natural coagulation and must be slightly centrifuged after retraction of the coagulum

(3) Titration time is at the minimum 24 hours after placing the tubes in the incubator and 26 at the maximum if accurate results are to be obtained

(4) As is known the N/20 sodium hydroxide should be kept in small flasks completely filled, so as to avoid alteration by the atmospheric CO_2, or should be protected by some device

(5) Since the volume used is small it is advisable to use microburettes

(6) Make sure that soap or other residues do not pollute the receptacles

Evaluation The difference between the number of cubic centimeters of sodium hydroxide solution used in the first and second tubes determines the lipasic activity of the blood serum The lipase titer is that difference multiplied by ten

Example In the first tube 1 8 ml of N/20 sodium hydroxide were used and in the second 1 0 ml The difference is 0 8 and the lipase titer is 8 0

Oil Emulsion Seabra s Lipase Reagent is packaged in cartons containing 60 ampules of 5 ml of the oil emulsion (ampules in white wrapper) Before opening the ampule its contents must be completely homogenized by adequate shaking For each determination 4 ml are used When making several determinations at the same time the remainder of the material may be collected and used The excess should be discarded however and must not be kept for later use

Buffer Solution Every box of Seabra s Lipase Reagent contains one ampule containing more than 5 ml of the buffer solution (ampule in green wrapper) Five ml measured exactly are dissolved in 70 ml of distilled water The solution is kept in a flask ready for use at any time and is sufficient for 75 determinations

References

1 SABIN F R C R AUSTRIAN R S CUNNINGHAM & C A DOAN 1942 Studies in the maturation of myeloblasts into myelocytes and on amitotic cell division in the peripheral blood in subacute myeloblastic leucemia J Exptl Med 40 845

2 FIESSINGER N 1923 Les ferments des leucocytes en physiologie et thérapeutique générale Masson Paris France

3 LOELE W 1936 Eine Dauerfarbung der Oxydasen in myeloischen Leukozyten im Blutausstrich Deut med Wochschr 62 2004

4 AMMON R 1936 Die Fermente unter pathologischen Bedingungen Deut med Wochschr 62 1958

5 SEABRA P 1939 Índice de oxidase Bol acad nacl med 3 9

6 LESSANHA J J & W LINS FILHO 1942 Índice de oxidase Seabra no diabetes mellitus Imprensa Médica 19 360

7 COELHO O & W LINS FILHO 1943 Índice de oxidase Seabra na fisiologia do vôo de altitude Imprensa Médica 19 360

8 ECHAVE D 1944 Relação entre o índice de oxidase Seabra e a cupremia normal e patológica Imprensa Médica 20 362

9 LINS FILHO W & M DE MAGALHAES PRADO 194 Índice de oxidase Seabra no trabalho mental O Hospital 27 4

10 SOUTO MAIOR M C 1946 Oxidase and diabetes Medical Times 7 4

taining a ribose sugar is called ribonucleic acid (RNA) The histochemical characterization of nucleic acid is possible in various ways

(a) Ribonucleic Acids (RNA)

RNA is found in both the nucleolus and cytoplasm and its identification is based on the fact that the strong basophilia can be selectively abolished by the specific depolymerizing action of ribonuclease For the demonstration of basophilia many acid dyes can be used However the methyl green pyronine technique is particularly suitable since it permits the simultaneous demonstration of RNA in cytoplasm and nucleolus as well as of desoxyribonucleic acid in the nuclear chromatin It is therefore a useful stain for the demonstration of both RNA and DNA[2]

The cytoplasm of hematocytoblasts and myeloblasts is deeply basophilic The myeloblasts acquire azurophilic granules as they develop into myelocytes and later the specific granules appear together with rapid and great reduction in cytoplasmic basophilia The nuclei of the young basophilic cells contain distinct nucleoli which are also basophilic These nucleoli disappear gradually in the more mature cells simultaneously with the reduction of cytoplasmic basophilia The higher RNA content of the cytoplasm and of the nucleoli is characterized by the ability to pass through a series of mitotic divisions Hyperplasia with immaturity of the marrow is associated with higher values for both nucleic acids especially of RNA[4] The cytoplasmic basophilia of plasma cells and lymphocytes is abolished by ribonuclease and is therefore likewise due to RNA[5] These results are in good agreement with those obtained by quantitative ultraviolet microspectrographic method[6] as applied to the study of blood cells[7]

Neutrophilic granules[8] Auer bodies in leukemic cells[9] as well as toxic granulation in leukocytes from patients with infectious diseases[10] apparently contain RNA since they cannot be stained following ribonuclease treatment

Although not strictly a histochemical method certain information concerning the chemical composition of cellular structures including their nucleic acid content can be obtained by studying the alteration that occur in the binding of acid and basic dyes at various pH level The pattern of staining for instance displayed by the cytoplasmic ground substance of many white cells by methylene blue through the pH range of 12 3 3 9 is characteristic of ribonucleic acid In contrast basophilia due to acid mucopolysaccharides will not be extinguished until a pH of 1 9 is reached[11 12]

(b) Desoxyribonucleic Acid

Desoxyribonucleic acid (DNA) can be demonstrated with the Feulgen technique in which the aldehyde groups of the desoxyribose sugar are first freed by mild acid hydrolysis and then visualized with Schiff s reagent The staining reaction which is confined entirely to the nuclei is specific for desoxyribonucleic acid of high and low polymer type In contrast methyl green apparently stains desoxyribonucleic acid selectively but only if it is in a highly polymerized state[1 3] Loss of staining ability for methyl green therefore indicates de

HISTOCHEMISTRY OF LEUKOCYTES

By Max Wachstein

Department of Pathology St Catherine s Hospital Brooklyn N Y

Although the beginning of histochemistry dates back almost 100 years[1] many new techniques have been devised only within the last few years These new methods indeed can give information not obtainable by other means A given substance *e g* may be present in such small amounts as to escape detection by biochemical estimation in tissue homogenates The presence of this substance however can be easily detected in a few cells of the same organ by appropriately staining a tissue section

Some histochemical procedures applicable to the study of blood cells will be discussed in this paper In applying these techniques to hematology the following points should be taken into account

(1) Histochemical localization within preformed structures of the cell may be incorrect This is particularly true in the case of the nucleus which may show a faulty staining reaction due to unspecific absorption On the other hand the lack of reaction in the nucleus may be due to the impermeability of the nuclear membrane for the reagents used in the staining procedure

(2) Reported findings obtained with histochemical techniques applied to the same tissues by various investigators are often at variance with each other in some detail Minor differences however should be neglected since variations in the preparation of the material as well as other technical details may influence the results obtained

(3) The tendency to subjective quantitation is noted in many publications Only for a few techniques *e g* the Feulgen stain are methods for a comparative quantitative evaluation of color available In general however histochemical staining techniques give only rough qualitative but no quantitative information

The observations recorded in this paper pertain mostly to films made from blood bone marrow and lymph nodes and from normal human material as well as from patients with blood dyscrasias and other diseases In the case of some sensitive enzymes *e g* cytochrome C and dehydrogenase cell suspensions from heparinized blood or bone marrow were used Staining reactions observed in frozen or paraffin sections from either bone marrow or lymph nodes often vary somewhat from those in film preparations

RESULTS

Nucleoproteins

The intense basophilia of immature blood cell has been known for many years The recognition that this staining property is due to two kinds of nucleic acids which in tissues are present in combination with basic proteins is of much more recent origin Nucleic acids consist essentially of purine and pyrimidine bases a pentose sugar and phosphoric acid The one containing desoxyribose is called desoxyribonucleic acid (DRA) whereas the other con

taining a ribose sugar, is called ribonucleic acid (RNA). The histochemical characterization of nucleic acid is possible in various ways.

(a) *Ribonucleic Acids (RNA)*

RNA is found in both the nucleolus and cytoplasm and its identification is based on the fact that the strong basophilias can be selectively abolished by the specific depolymerizing action of ribonuclease.[2] For the demonstration of basophilia many acid dyes can be used. However, the methyl green pyronine technique is particularly suitable since it permits the simultaneous demonstration of RNA in cytoplasm and nucleolus as well as of desoxyribonucleic acid in the nuclear chromatin. It is, therefore, a useful stain for the demonstration of both RNA and DNA.[3]

The cytoplasm of hematocytoblasts and myeloblasts is deeply basophilic. The myeloblasts acquire azurophilic granules as they develop into myelocytes and later the specific granules appear together with rapid and great reduction in cytoplasmic basophilia. The nuclei of the young basophilic cells contain distinct nucleoli which are also basophilic. These nucleoli disappear gradually in the more mature cells, simultaneously with the reduction of cytoplasmic basophilia. The higher RNA content of the cytoplasm and of the nucleoli is characterized by the ability to pass through a series of mitotic divisions. Hyperplasia with immaturity of the marrow is associated with higher values for both nucleic acids, especially of RNA.[4] The cytoplasmic basophilia of plasma cells and lymphocytes is abolished by ribonuclease and is therefore likewise due to RNA.[5] These results are in good agreement with those obtained by quantitative ultraviolet microspectrographic method,[6] as applied to the study of blood cells.[7]

Neutrophilic granules,[8] Auer bodies in leukemic cells,[9] as well as toxic granulation in leukocytes from patients with infectious diseases[10] apparently contain RNA, since they cannot be stained following ribonuclease treatment.

Although not strictly a histochemical method, certain information concerning the chemical composition of cellular structures, including their nucleic acid content, can be obtained by studying the alterations that occur in the binding of acid and basic dyes at various pH levels. The pattern of staining, for instance, displayed by the cytoplasmic ground substance of many white cells by methylene blue through the pH range of 12 3–3 9 is characteristic of ribonucleic acid. In contrast, basophilia due to acid mucopolysaccharides will not be extinguished until a pH of 1 9 is reached.[11, 12]

(b) *Desoxyribonucleic Acid*

Desoxyribonucleic acid (DNA) can be demonstrated with the Feulgen technique in which the aldehyde groups of the desoxyribose sugar are first freed by mild acid hydrolysis and then visualized with Schiff's reagent. The staining reaction, which is confined entirely to the nuclei, is specific for desoxyribonucleic acid of high and low polymer type. In contrast, methyl green apparently stains desoxyribonucleic acid selectively, but only if it is in a highly polymerized state.[13, 14] Loss of staining ability for methyl green therefore indicates de

polymerization Both staining techniques can be used in a comparative quantitative way permitting measurements of single cells with a microspectrophotometric apparatus[15] The applicability of these quantitative techniques for blood cells has been shown by Korson[16]

With the Feulgen method the nuclei of immature cells have a relatively pale coloration Mature white cells usually have more deeply tinged nuclei Although the myeloblastic nucleus takes a pale stain the chromatin pattern can be clearly distinguished Within it, two to four nuclei are detectable as colorless spots Lymphoblasts show a fine structure of the Feulgen material and two nucleoli while mature lymphocytes have coarse Feulgen positive chromatin[17] Comparative quantitative measurements have revealed a decrease of DNA during the development of the myeloid cells[18] For normal lymphocytes very constant values were found by Petrakis[19] The DNA content of the normal lymphocytes averages twice that of spermatides In chronic lymphatic leukemia, the lymphocytes have mostly a normal DNA content However in occasional cases with acute exacerbation, cells were found which showed an irregular increase in DNA probably due to increased mitotic activity or polyploidy and polyteny Similarly Petermann and Schneider[20] have found on chemical examination a normal DNA content in isolated nuclei from the spleen of mice in slowly developing spontaneous leukemia In contrast there was a marked increase of both the RNA and DNA in the nuclei from the spleen of mice with the more rapidly developing transplanted leukemia These changes are characteristic of rapid growth rather than of the neoplastic process *per se*

Proteins

Staining reactions are available for the demonstration of basic proteins as well as for amino acid components of proteins

(a) *Basic Proteins*

A selective staining method for the basic proteins of cell nuclei has been reported by Alfert and Geschwind[21] The method depends upon the controlled binding of the acid dye fast green by basic proteins at a pH of more than 8 In tissues these are chiefly histones protamines and to a slight extent, globins This means that ordinarily only nuclear chromatin where histones are located will stain and that red blood corpuscles are colored faintly

Bloch and Godman[22] have recently shown that this method can be used for comparative microspectrophotometric measurements In addition they have found that the same cell can be first stained with the Feulgen technique measured for its DNA content and then after washing out the Fuchsin be restained with fast green for measurement of the histone protein This method is also applicable to the study of the basic protein of blood cell nuclei It would appear that the nuclei of the white cells as has been found by Bloch and Godman for other tissues have an identical distribution of DNA and its associated basic proteins

(b) *Amino Acids*

A number of reactions are available for the histochemical identification of amino acid components of proteins[1, 21] At least four techniques are suitable for hematological purposes These four comprise two older methods for tyrosine and arginine and two more recent ones for free amino and SH groups

The Millon reaction is specific for phenols and in the case of amino acids for tyrosine Pollister s modification[18] is best suited for hematological purposes The appearance of a transparent pink color indicates the presence of tyrosine Pollister reported positive staining of eosinophilic granules in tissue sections from guinea pigs while Vercauteren had negative results in eo inophilic leukocytes from the horse[21] We found a positive reaction of eosinophilic granules but no staining in other blood cells in human material

Sakaguchi s test is specific for derivatives of guanidine Arginine is the only substance found in tissues which gives a positive reaction The technique of Serra or Baker's modification of this procedure may be used[22] According to Weiss the nuclei of all blood and bone marrow cells are colored a faint to moderate orange The reaction in the cytoplasm of myeloid cells varies in intensity Neutrophilic granules do not react while eos nophilic granules stain distinctly[12]

The demonstration of primary amino groups in proteins is based on the fact that these groups react with aldehydes to form Schiff bases The reagent used in this technique o hydroxy 2 naphthyl aldehyde forms a pale yellow addition product The latter is transformed into a red or blue azo dye[25] With this technique a faint pink staining was found in all blood and bone marrow cells In general the cytoplasm stained somewhat more intensely than the nucleus Megakaryocytes showed the most distinct nuclear staining Neutrophilic granules were unstained but eosinophilic granules showed a brilliant yellow pink color

Active sulfhydryl groups react with 2,2-dihydroxy-6-dinaphthyl to form a colorle s substance which can be converted into a colored azo dye[26] Barnett reported staining of bone marrow cells in human tissue sections[27] In films from blood and bone marrow a distinct pink to red stain was seen in most cell In general the cytoplasm was more intensely colored In many cells however the chromatin network was also outlined No reaction was seen in either neutrophilic or eosinophilic granules No obvious increase in staining intensity of leukemic as compared with normal myeloid elements was noticed On chemical estimation an increase in leukocytic SH content was reported in patients with various forms of leukemias[28] These findings however were not confirmed by Valentine[23]

Lipids

Oil soluble dyes particularly Sudan black stain certain structures in most white blood cell There exists however a considerable difference between lipids in leukocytes and in other tissues Tissue lipid stain easily at room temperature but with blood cells it is necessary to use longer staining periods

or higher temperatures. Decolorization is difficult and recolorization is often incomplete. In contrast, tissue lipids stained with Sudan are promptly decolorized by dye solvents and can be restained and recolorized repeatedly. The sudanophilia of leukocytes probably depends on a chemical combination of the dyes with cytoplasmic constituents.[30] Following the exposure of blood and bone marrow cells to Sudan black, myeloblasts show small stained inclusions. With the maturation of myeloid cells sudanophilia increases markedly. Granules in neutrophiles are prominently stained and appear to be more numerous than those seen with Giemsa stains. Eosinophilic granules are strongly positive and show often an unstained hollow center. While the basophilic granulations show a varying reaction, monoblasts as well as monocytes show small stained granules. Auer bodies in leukemic cells are Sudan positive.[9] Plasma cells show occasional lipid granules.[31, 3] The application of Baker's method for phospholipids shows an essentially similar distribution of the staining reaction. However, in immature blood cells, small rods and granules, presumably mitochondria, are visualized with Baker's method to a much greater degree than with Sudan black.[31]

While most investigators found that mature lymphocytes give no reaction with fat stains, occasional refractile bodies were reported to stain with Sudan black and Nile blue sulfate when the preparation of the blood films was somewhat modified.[32] In general, under pathological conditions no significant changes in the lipid staining of white cells was found. In chronic myeloid leukemia and infectious diseases, however, a small proportion of cells may not contain stainable fat.[32, 33]

Glycogen

Glycogen was first demonstrated by Ranvier in 1877 in the white cells of the frog with the help of the iodine reaction.[34] Best results are obtained by the use of the Periodic Acid Schiff technique.[1, 23]

Periodic acid acts as an oxidant which breaks the C—C bonds where they are present as 1,2 glycol groups and converts them into dialdehydes. The latter are then visualized with Schiff's reagent. In order to identify the stained material as glycogen, control films must be exposed to the action of ptyalin (saliva). Any material giving a positive reaction after salivary digestion is considered not to be glycogen. There is conflicting evidence in the literature as to the effect of this procedure on white blood cells.[1] It appears, however, that in films fixed in absolute alcohol, but not in air-dried unfixed films, the stainable substance is removed by salivary digestion. It is probable that alcohol fixation frees glycogen from some chemical combination, possibly with protein.[35]

About 90 per cent of polymorphonuclear leukocytes contain stainable material, mostly distributed diffusely in the cytoplasm, but occasionally in the form of coarse deposits not localized in the neutrophilic granulations. Eosinophiles and basophiles[36] show likewise cytoplasmic but no granular staining. Glycogen is present in plasma cells and myeloma cells as well as in monocytes and lymphocytes to a varying degree.[35, 37, 38, 39] Myeloblasts contain only

small amounts of stainable glycogen As the cells mature the staining intensity increases in bone marrow films prepared from normal individuals and from patients with chronic myeloid leukemia[35]

Histochemical methods have revealed significant differences in the staining intensity of polymorphonuclear leukocytes in various conditions A marked increase was found in the blood of patients with infectious diseases[35 40] in polycythemia vera,[38] and in severe diabetes[41] While no significant difference in glycogen distribution was found in cells from normal subjects and patients with chronic myeloid or lymphatic leukemia[35 38] several investigators reported an occasional increase in lymphocytes in blood smears[37] and tissue sections[42] By chemical estimation high values in leukocytic glycogen were found in polycythemia[43 44] and low values in chronic lymphatic and acute leukemia[43] In chronic myeloid leukemia the reported results were less uniform[43 44]

Enzymes

Only a limited number of oxidative and hydrolytic enzymes can be demonstrated histochemically[1 23] Some of these are found in blood cells

(A) *Oxidative Enzymes*

(1) *Oxidases* The M Nadi reaction and peroxidase reaction which is limited to the granules of myeloid cells and is resistant to formalin and other fixatives has been discussed by Seabra[45]

Reference will be made here only to cytochrome oxidase (labile or G Nadi reaction) This enzyme is quite sensitive to various procedures even drying It is present in many tissues but is not very extensively found in white blood cells It is visualized with the help of the Nadi reaction which consists of the simultaneous oxidation of a mixture of dimethyl p-phenylene diamine and alpha naphthol and subsequent condensation of the initial oxidation product insoluble indophenol blue being the end product While the reaction appears to proceed spontaneously in the presence of molecular oxygen (auto-oxidation) its rate is greatly increased by the presence of indophenol oxidase which is presumably identical with Keilin s cytochrome oxidase

In lymphocytes from normal lymph nodes as well as from lymph nodes with leukemia lymphosarcoma and Hodgkin s disease 5 to 20 blue granules are visualized indicating sites of enzymatic activity No difference in the staining reaction was seen in material from any of these sources[46] In myeloid cells the reaction is observed as myelocytes mature into polymorphonuclear leukocytes Monocytes show considerable activity In agreement with findings obtained on tissue extracts with biochemical estimation no difference was found in the staining reaction of leukemic and nonleukemic cells[47] In inflammatory exudates the reaction is distinctly stronger not only in neutrophiles but also in lymphocytes[48]

(2) *Dehydrogenases* Dehydrogenases catalyze the transfer of hydrogen to immediate acceptors other than oxygen For their demonstration one can use potassium tellurite which is reduced to insoluble black elementary tellurium[49] However reagents best suitable for the demonstration of these

enzymes are the tetrazolium salts These salts are colorless water soluble substances which form highly colored water insoluble precipitates after they have accepted hydrogen

If cells from blood, from bone marrow, or from lymph nodes are incubated in a solution of neotetrazolium buffered at pH 7 4 a considerable portion of cells show evidence of reducing activity as indicated by the deposition of small purplish granules in their cytoplasm[60] Still better results were obtained in occasional blood samples if the incubation mixture contained in addition, various activators as well as sodium succinate[61]

Dehydrogenase activity is found in approximately three fourths of all white blood cells in films from normal individuals Polymorphonuclear leukocytes, lymphocytes and monocytes contain a varying amount of purplish granules indicating the site of reducing activity These granules are independent from the specific granulations Evidence of enzymatic activity is noted first in myelocytes while myeloblasts do not react In blood from patients with infectious diseases, the cells contain more and often bigger granules, and the same phenomenon was seen in cells from inflammatory exudates As with cytochrome C there was no significant difference in cells from blood and bone marrow from patients with myeloid leukemia as compared with those from normal controls

✓ (B) *Hydrolytic Enzymes*

(1) *Phosphatases* Phosphatases are enzymes which are able to hydrolyze phosphoric esters The phosphatases may be classified on the basis of substrate specificity and of pH optima

(1a) *Nonspecific alkaline phosphatases* This phosphatase hydrolyzes many monoesters of phosphoric acids and in addition nucleic acids Two techniques are at the present available for their histochemical demonstration[1, 23]

(i) *The calcium phosphate method* The slides are incubated with glycerophosphate at an alkaline reaction in the presence of calcium The liberated phosphate ions are precipitated as insoluble calcium phosphate The latter is visualized as metallic silver or black cobalt sulfide

(ii) *The azo dye method* The substrate contains a naphthyl phosphate The liberated naphthol is coupled with a suitable diazonium salt to form an insoluble colored precipitate

Using a somewhat modified calcium phosphate technique normal individuals show moderate activity in about one third of the circulating neutrophiles[62] The staining is seen mostly in the cytoplasm and occasionally also in the nuclei especially after long incubation periods Circulating lymphocytes monocytes and eosinophiles show usually a negative reaction Bone marrow preparations from normal individuals show no reaction in myeloblasts a faint reaction in myelocytes, and increasing staining intensity as the cells mature A significantly higher proportion of positive myeloid cells was found in films from bone marrow than in films from peripheral blood Similarly more positive reacting lymphocytes were noticed in film prepared from lymph nodes than in blood films

Striking differences in the content of nonspecific alkaline phosphatase are present in various abnormal conditions In patients with infectious diseases more than 90 per cent of all neutrophiles show a positive reaction In addition the staining intensity is considerably increased in these cells as well as in polymorphonuclear leukocytes found in inflammatory exudates [52] In contrast in patients with chronic myeloid leukemia only a few and in some cases no phosphatase positive mature polymorphonuclear leukocytes were found [52,53] On the other hand in patients with myelofibrotic disorders and a leukemoid blood picture many phosphatase positive granulocytes are present [6] These results are in good agreement with those found by biochemical estimation since high values for nonspecific alkaline phosphatase are found in the white blood cells of patients with infectious diseases and with myelofibrotic disorders In contrast there is only a small amount of this enzyme present in the leukocytes of patients with various forms of leukemia including the chronic myeloid form [44,54,55]

The nuclear localization which is occasionally seen when the calcium phosphatase technique is used does not represent enzymatic activity but is apparently due to a staining artifact [1,23] Even very recent publications stressing differences in supposed nuclear localization disregard this now well established fact [55] If the azo dye method in Gomori s modification [3] is applied to blood and bone marrow smears nuclear staining is not seen

(1b) *Acid phosphatases* As in the case of the alkaline phosphatase two techniques are available for acid phosphatase [1,23]

(i) *Gomori s lead phosphate method* Slides are incubated in a mixture containing glycerophosphate at an acid pH in the presence of lead ions Liberated phosphate is precipitated as lead salt and the latter is transformed into black lead sulfide

(ii) *The a o dye method* This technique is similar in principle to the technique for nonspecific alkaline phosphatase Recently Rutenburg and Seligman have introduced a new substrate 6-benzoyl 2 naphthyl phosphate with which sites of activity are stained very brilliantly [56]

In films from blood and bone marrow stained with the lead phosphate method there is seen a moderate cytoplasmic and very distinct nuclear reaction in all cells of the red and white series [57,58] A positive reaction in addition was noticed in occasional neutrophilic granules and regularly in eosinophilic granules Mast cells also react positive [59] There is some doubt whether nuclear staining with the lead technique indicates enzymatic activity or is due to staining artefacts [1,23] For blood cells this point has been discussed by Mori [60]

In attempts to evaluate the results obtained with the lead phosphate technique smears from blood and bone marrow were stained with the azo dye technique using various substrates including the recently reported 6-benzoyl 2 naphthyl phosphate No satisfactory preparations so far have been obtained As in the case of alkaline phosphatase reported differences in nuclear staining for nonspecific acid phosphatase [42,55] will have to be re-evaluated

(1c) *5 Nucleotidase* The existence of a specific enzyme 5 nucleotidase

enzymes are the tetrazolium salts These salts are colorless water soluble substances which form highly colored water insoluble precipitates after they have accepted hydrogen

If cells from blood, from bone marrow, or from lymph nodes are incubated in a solution of neotetrazolium buffered at pH 7 4, a considerable portion of cells show evidence of reducing activity as indicated by the deposition of small purplish granules in their cytoplasm[60] Still better results were obtained in occasional blood samples if the incubation mixture contained in addition, various activators as well as sodium succinate[61]

Dehydrogenase activity is found in approximately three fourths of all white blood cells in films from normal individuals Polymorphonuclear leukocytes, lymphocytes and monocytes contain a varying amount of purplish granules indicating the site of reducing activity These granules are independent from the specific granulations Evidence of enzymatic activity is noted first in myelocytes while myeloblasts do not react In blood from patients with infectious diseases the cells contain more and often bigger granules and the same phenomenon was seen in cells from inflammatory exudates As with cytochrome C there was no significant difference in cells from blood and bone marrow from patients with myeloid leukemia as compared with those from normal controls

(B) *Hydrolytic Enzymes*

(1) *Phosphatases* Phosphatases are enzymes which are able to hydrolyze phosphoric esters The phosphatases may be classified on the basis of substrate specificity and of pH optima

(1a) *Nonspecific alkaline phosphatases* This phosphatase hydrolyzes many monoesters of phosphoric acids and in addition nucleic acids Two techniques are at the present available for their histochemical demonstration[1,23]

(i) *The calcium phosphate method* The slides are incubated with glycerophosphate at an alkaline reaction in the presence of calcium The liberated phosphate ions are precipitated as insoluble calcium phosphate The latter is visualized as metallic silver or black cobalt sulfide

(ii) *The azo dye method* The substrate contains a naphthyl phosphate The liberated naphthol is coupled with a suitable diazonium salt to form an insoluble colored precipitate

Using a somewhat modified calcium phosphate technique normal individuals show moderate activity in about one third of the circulating neutrophiles[62] The staining is seen mostly in the cytoplasm and occasionally also in the nuclei, especially after long incubation periods Circulating lymphocytes monocytes, and eosinophiles show usually a negative reaction Bone marrow preparations from normal individuals show no reaction in myeloblasts a faint reaction in myelocytes and increasing staining intensity as the cells mature A significantly higher proportion of positive myeloid cells was found in films from bone marrow than in films from peripheral blood Similarly more positive reacting lymphocytes were noticed in film prepared from lymph nodes than in blood films

Striking differences in the content of nonspecific alkaline phosphatase are present in various abnormal conditions In patients with infectious diseases more than 90 per cent of all neutrophiles show a positive reaction In addition the staining intensity is considerably increased in these cells as well as in polymorphonuclear leukocytes found in inflammatory exudates[52] In contrast in patients with chronic myeloid leukemia only a few and in some cases no phosphatase positive mature polymorphonuclear leukocytes were found[52, 53] On the other hand in patients with myelofibrotic disorders and a leukemoid blood picture many phosphatase positive granulocytes are present[4] These results are in good agreement with those found by biochemical estimation since high values for nonspecific alkaline phosphatase are found in the white blood cells of patients with infectious diseases and with myelofibrotic disorders In contrast there is only a small amount of this enzyme present in the leukocytes of patients with various forms of leukemia including the chronic myeloid form[44, 54, 55]

The nuclear localization which is occasionally seen when the calcium phosphatase technique is used does not represent enzymatic activity but is apparently due to a staining artifact[1, 23] Even very recent publications stressing differences in supposed nuclear localization disregard this now well-established fact[54] If the azo dye method in Gomori s modification[23] is applied to blood and bone marrow smears nuclear staining is not seen

(1b) *Acid phosphatases* As in the case of the alkaline phosphatase two techniques are available for acid phosphatase[1, 23]

(i) *Gomori s lead phosphate method* Slides are incubated in a mixture containing glycerophosphate at an acid pH in the presence of lead ions Liberated phosphate is precipitated as lead salt and the latter is transformed into black lead sulfide

(ii) *The azo dye method* This technique is similar in principle to the technique for nonspecific alkaline phosphatase Recently Rutenburg and Seligman have introduced a new substrate 6-benzoyl 2 naphthyl phosphate with which sites of activity are stained very brilliantly[56]

In films from blood and bone marrow stained with the lead phosphate method there is seen a moderate cytoplasmic and very distinct nuclear reaction in all cells of the red and white series[57, 58] A positive reaction in addition was noticed in occasional neutrophilic granules and regularly in eosinophilic granules Mast cells also react positive[59] There is some doubt whether nuclear staining with the lead technique indicates enzymatic activity or is due to staining artefacts[1, 23] For blood cells this point has been discussed by Mori[60]

In attempts to evaluate the results obtained with the lead phosphate technique smears from blood and bone marrow were stained with the azo dye technique using various substrates including the recently reported 6-benzoyl 2 naphthyl phosphate No satisfactory preparations so far have been obtained As in the case of alkaline phosphatase reported differences in nuclear staining for nonspecific acid phosphatase[42, 55] will have to be re-evaluated

(1c) *5 Nucleotidase* The existence of a specific enzyme 5 nucleotidase

give positive reactions with both techniques [21, 65] Under certain experimental conditions occasional histiocytes acquire histochemically demonstrable esterase activity [66] Hematic elements are otherwise negative with both substrates

Gomori has shown recently that chloracylesters are suitable substrates for leukocytic esterase [67] He made u e of the chloracetate of alpha naphthol and naphthol AS With both these substrates mast cells are intensely positive In addition myeloid elements give positive reactions The staining involves all stages of maturation from myelocytes to mature polymorphonuclear leukocytes Myeloblasts monocytes and lymphocytes show no reaction Al though some of the myeloid cells do not stain a deficiency in this enzyme in mature cells of chronic myeloid leukemia similar to the one found for non specific alkaline phosphatase is not observed

Recently acetylcholin esterase was demonstrated in blood and bone marrow cells with a modification of Koelle s technique [67] Suspensions of blood and bone marrow cells were incubated in a medium containing acetylthiocholin and copper glycinate The enzymatically liberated thiocholin forms crystals of copper thiocholine at sites of enzymatic activity These crystals can be seen in the phase microscope

In human subjects evidence of acetylcholin esterase activity was found in megacarocytes and red cells and in rats and rabbits in megacaryocytes but in no other blood cells These findings agree well with previously established results with biochemical measurements on these cells and confirm the absence of this enzyme in white cells

Beta Glucoronidase

The beta glucoronidases are a group of enzymes with a pecificity for the beta glyco ide linkage of a variety of naturally occurring and synthetic glu coronides Of the histochemical methods described two were tried on films of blood and bone marrow

The method II of Friedenwald and Baker [69] depends on the hydrolysis of 8-hydroxyquinoline glucoronide in the presence of a ferric salt and the subse quent visualization of the formed ferric hydroxyquinoline as Prussian blue Even in the modification of Pierce and Burton [1] no satisfactory staining results were obtained Seligman and his co-workers[70] have reported a new method using a synthetic 6-bromo 2 naphthyl beta D glucusonide with ubsequent vi ualization of the naphthol component as an azo dye We obtained very satisfactory results with this method in kidney sections If frozen sections from rat spleen as described by Seligman and his co-workers or sections from human tonsils were used marked staining of lymph follicles resulted Under high power magnification however the dye could not be localized within lymphatic cells No staining was ob erved in any of the nucleated cells in blood or bone marrow smears

By biochemical methods measurable beta glucoronidase activity has been found in leukocytes of normal ubjects and marked variations under abnormal conditions [71] This discrepancy between the results of biochemical and histochemical methods warrants further investigation

capable of hydrolyzing nucleotides, for instance muscle adenylic acid which contain phosphate esterified at carbon 5 of ribose has been first reported by Reis in various tissue extracts by chemical estimation[61] Its optimal activity is around a pH of 8 It can be demonstrated with a similar technique as non specific alkaline phosphatase, using muscle adenylic acid as substrate[23, 62] A nucleotidase which however has its pH optimum at pH 4 has been reported to occur in leukocytes on chemical estimation[63]

On incubating blood and bone marrow smears with both glycerophosphate and muscle adenylic acid at a pH of 9 enzymatic activity was found with both substrates At a pH of 8 however the staining was seen only with glycerophosphate but not with muscle adenylic acid This observation would indicate that any staining reaction obtained was due to the presence of non pecific alkaline phosphatase which also acts on muscle adenylic acid at a higher degree of alkalinity

(1d) *Glucose 6 phosphatase* This enzyme is important for the breakdown of glycogen to glucose It can be distinguished from nonspecific phosphatase by its thermal instability its pH optimum (pH 6 5–6 7) and its sensitivity to various fixatives including formalin A histochemical method based on the liberation of phosphate from glucose 6 phosphate at pH 6 7 in the presence of lead ions at 32° C was recently reported by Chiquoine[64]

In applying this method to unfixed blood and bone marrow smears, we have observed the appearance of granular precipitates of lead sulfide indicating enzymatic activity in the cytoplasm of various myeloid cells Formalin fixation however did not prevent this staining reaction Moreover control slides, either fixed or unfixed incubated with glycerophosphate instead of glucose 6-phosphate, showed a similar staining pattern The predominant cytoplasmic localization at a higher pH, as compared to the almost exclusive nuclear staining around pH 5 with the lead phosphate technique, is of considerable interest[84] On the basis of these findings it would appear that glucose 6 phosphatase can not be distinguished histochemically from nonspecific phosphatase in white blood cells

Esterases

Esterases are enzymes which hydrolyze esters of carboxylic acids They can be divided into aliesterases comprising both lipases and unspecific esterases and in choline esterases

For the demonstration of aliesterases two techniques are available[1, 3]

(a) *The Tween Technique*

The substrates are Tweens or similar substances which consist of water soluble fatty esters of sorbitan or mannitan Liberated fatty acids are precipitated in the form of insoluble calcium salts and visualized as in the technique for acid phosphatase

(b) *The Azo Dye Method*

Various naphthyl acetates may be used as substrates The liberated naphthol is visualized by coupling with a suitable diazonium salt Only mast cells

give positive reactions with both techniques[3, 63] Under certain experimental conditions occasional histiocytes acquire histochemically demonstrable esterase activity[66] Hematic elements are otherwise negative with both substrates

Gomori has shown recently that chloracylesters are suitable substrates for leukocytic esterase[67] He made use of the chloracetate of alpha naphthol and naphthol AS With both these substrates mast cells are intensely positive In addition myeloid elements give positive reaction The staining involves all stages of maturation from myelocytes to mature polymorphonuclear leukocytes Myeloblasts monocytes and lymphocytes show no reaction Although some of the myeloid cells do not stain a deficiency in this enzyme in mature cells of chronic myeloid leukemia similar to the one found for non specific alkaline phosphatase is not observed

Recently acetylcholin esterase was demonstrated in blood and bone marrow cells with a modification of Koelle's technique[67] Suspensions of blood and bone marrow cells were incubated in a medium containing acetylthiocholin and copper glycinate The enzymatically liberated thiocholin forms crystals of copper thiocholine at sites of enzymatic activity These crystals can be seen in the phase microscope

In human subjects evidence of acetylcholin esterase activity was found in megacarocytes and red cells and in rats and rabbits in megacaryocytes but in no other blood cells These findings agree well with previously established results with biochemical measurements on these cells and confirm the absence of this enzyme in white cells

Beta Glucoronidase

The beta glucoronidases are a group of enzymes with a specificity for the beta glycoside linkage of a variety of naturally occurring and synthetic glucoronides Of the histochemical methods described two were tried on films of blood and bone marrow

The method II of Friedenwald and Baker[69] depends on the hydrolysis of 8-hydroxyquinoline glucoronide in the presence of a ferric salt and the subsequent visualization of the formed ferric hydroxyquinoline as Prussian blue Even in the modification of Pierce and Burton[1] no satisfactory staining results were obtained Seligman and his co-workers[70] have reported a new method using a synthetic 6-bromo 2 naphthyl beta D glucuronide with subsequent visualization of the naphthol component as an azo dye We obtained very satisfactory results with this method in kidney sections If frozen sections from rat spleen as described by Seligman and his co-workers or sections from human tonsils were used marked staining of lymph follicles resulted Under high power magnification however the dye could not be localized within lymphatic cells No staining was observed in any of the nucleated cells in blood or bone marrow smears

By biochemical methods measurable beta glucoronidase activity has been found in leukocytes of normal subjects and marked variations under abnormal conditions[71] This discrepancy between the results of biochemical and histochemical methods warrants further investigation

Concluding Remarks

Interesting results have been obtained by the application of histochemical methods to the study of white blood cells Ribonucleic acid, for instance is present in large amounts in young cells and is obviously connected with their multiplication It diminishes as cells reach their functional maturity In contrast other substances for instance glycogen and various enzymes such as nonspecific alkaline phosphatase accumulate as the cells mature and are further increased in leukocytes which are stimulated to the highest pitch of functional activity under the influence of an infectious disease On the other hand the marked decrease or even complete absence of nonspecific alkaline phosphatase in the mature granulocytes of patients with chronic myelocytic leukemia is apparently the only microscopically demonstrable reaction which distinguishes mature leukemic cells from normal granulocytes

Many chemical reactions known to occur in biological material await adaptation for histochemical purposes But even by wider application of methods already available at present to various problems of hematology useful and interesting results can be anticipated

It is important to realize that histochemical methods furnish valuable tools for those engaged in morphologic research for the achievement of their ultimate goal namely the interpretation of structure in terms of biological function

Bibliography

1 Pearse E A G 1953 Histochemistry Theoretical and Applied Little Brown & Co Boston Mass
2 Brachet J 1940 *La detection histochimique des acides pentose nucléiques* Compt rend soc biol 133 88
3 White J C 1947 The cytoplasmic basophilia of the bone marrow cell J Path Bact 59 223
4 Davidson J N I Leslie & J C White 1948 The cytoplasmic basophilia of marrow cells the distribution of nucleic acids J Path Bact 60 1
5 Rheingold J J & G B Wislocki 1948 Histochemical method applied to hematology *Blood* 3 641
6 Caspersson T O 1950 Cell Growth and Cell Function a Cytochemical Study Norton New York N Y
7 Thorell B 1947 Studies on the formation of cellular substances during blood cell production Acta Med Scand Suppl 200
8 Berghe L & J Hofman 1945 *Présence d acide ribonucléique dans les granulations neutrophiles des leukocytes de l homme* Compt rend soc biol 139 324
9 Ackerman G A 1950 *Microscopic and histochemical studies on the Auer bodies in leukemic cells* Blood 5 847
10 Jackson J F 1954 The cytology of the degenerating leukocyte J Lab Clin Med 43 227
11 Dempsey E W H Bunting M Singer & G B Wislocki 1947 The dye binding *capacity and other chemo-histological properties of mammalian mucopolysaccharides* Anat Record 98 417
12 Weiss L P 1953 Binding of acid and basic dye at various Ph by blood and bone marrow cells of man with observation of blood and bone marrow stained with Serra s method for arginine Blood 8 249
13 Kurnick N B 1952 *The basis of specificity of methylgreen staining* Exptl Cell Research 3 649
14 Vercauteren R 1951 The structure of desoxyribose nucleic acid in relation to the cytochemical significance of the methylgreen pyronin staining Enzymologia 14 135
15 Pollister A 1950 Quelques méthodes de cytologie chimique quantitative Rev Hematol 5 527

16 KORSON R 1951 A microspectrophotometric study of red cell nuclei during pyknosis J Exptl Med 93 121
17 GARDIKAS C & M C G ISRAELS 1940 The Feulgen reaction applied to clinical hematology J Clin Path 1 226
18 MARINONE G 1951 Études cytophotométriques sur la teneur en acide desoxyribonucléique des cellules myeloides et lymphoides au cours de leur evolution et reproduction Rev belge path et méd 21 332
19 PETRAKIS N L 1953 Microspectrophotometric estimation of the desoxyribonucleic acid (DNA) content of individual normal and leukemic human leukocytes Blood 8 905
20 PETERMAN M L & R SCHNEIDER 1951 Nuclei from normal and leukemic mouse spleen II The nucleic acid content of normal and leukemic nuclei Cancer Research 11 485
21 ALFERT M & I I GESCHWIND 1953 A selective staining method for the basic proteins of cell nuclei Proc Natl Acad Sci U S 39 991
22 BLOCH D P & G C GODMAN 1954 A microspectrophotometric study of DNA and basic proteins of the nucleus of the mitotic cycle Proc 5th Ann Meet Histochemical Soc J Histochem and Cytol 2 455
23 GOMORI G 1952 Microscopic Histochemistry Principles and Practice Univ of Chicago Press Chicago Ill
24 VERCAUTEREN R 1951 A cytochemical approach to the problem of the significance of blood and tissue eosinophilia Enzymologia 14 340
25 WEISS L P K C TSOU & A M SELIGMAN 1954 Histochemical demonstration of protein bound amino groups J Histochem Cytochem 2 29
26 BARNETT R J & A M SELIGMAN 1952 Histochemical demonstration of protein bound sulfhydryl groups Science 116 323
27 BARNETT R J 1953 The histochemical distribution of protein bound sulfhydryl groups J Natl Cancer Inst 13 905
28 CONTOPOULAS A N & H H ANDERSON 1950 Sulfhydryl content of blood in dyscrasias J Lab Clin Med 36 924
29 VALENTINE W N 1955 The enzymes of leukocytes Ann N Y Acad Sci 59(5) 1003
30 LILLIE R D & A J BURTNER 1953 Stable sudanophilia of human neutrophil leukocytes in relation to peroxidase and oxidase J Histochem Cytochem 1 8
31 BLOOM M L & C B WISLOCKI 1950 The localization of lipids in human blood and bone marrow cells Blood 5 79
32 HAYHOE F G J 1953 The cytochemical demonstration of lipids in blood and bone marrow cells J Path Bact 65 413
32a HEMPLEMANN L H & N P KNOWLTON JR 1953 The nature of neutral red staining refractive particles in the lymphocytes of the blood of normal humans Blood 8 524
33 STORTI E & S PERUGINI 1950 Cytochemical researches on the lipids of the hematic cells and with particular attention to those of acute leucosis Acta hematol 6 321
34 RANVIER L 1877 Anatomie générale Progrès Méd cal Paris France
35 WACHSTEIN M 1949 The distribution of histochemically demonstrable glycogen in human blood and bone marrow cells Blood 4 54
36 SMITH C 1949 Glycogen in basophilic leukocytes in human blood smears Proc Soc Exptl Biol Med 72 209
37 WISLOCKI G B J J RHEINGOLD & E W DEMPSEY 1949 The occurrence of the periodic acid Schiff reaction in various normal cells of blood and connective tissue Blood 4 562
38 GIBB R P & R E STOWELL 1949 Glycogen in human blood cells Blood 4 569
39 ERANKÖ O 1950 Cytochemically demonstrable glycogen in human white blood cells Ann med exptl biol Fenn 28 1
40 MANCINI R E 1948 El glucogeno en los leucocitos de la sangre trasudades y exudados Rev soc argentina biol 24 249
41 MANCINI R E 1949 Variaciones del glucogeno de los neutrofilos en las diabetes humana Rev soc argentina biol 25 190
42 ACKERMAN G A R A KNOUFF & H A HOSTER 1951 Cytochemistry and morphology of neoplastic and non neoplastic human lymph node cells with special reference to Hodgkin s disease J Natl Cancer Inst 12 465
43 WAGNER R 1947 Studies on the physiology of the white blood cell The glycogen content of leukocytes in leukemia and polycythemia Blood 2 235

44 VALENTINE W N W S BECK J H FOLETTE H MILLS & J S LAWRENCE 1952 Biochemical studies in chronic myelocytic leukemia polycythemia vera and other idiopathic myeloproliferative disorders Blood 7 959
45 SEABRA P 1955 Oxidase and lipase of the leukocytes Ann N Y Acad Sci 59(5) 1022
46 HOFFMANN G T A ROTTINO & K G STERN 1951 Demonstration of the Nadi reaction of cytochrome oxidase activity in cells of the lymphoid and myeloid series obtained from normal individuals and patients suffering from Hodgkin s and other diseases Blood 6 1051
47 GREENSTEIN J P J WERNE A B ESCHENBRENNER & F M LEUTHARDT 1944 Chemical studies on human cancer I Cytochrome oxidase cytochrome c and copper in normal and neoplastic tissue J Natl Cancer Inst 5 55
48 REBUCK J W 1952 Enhanced nadireaction in lymphocytes in inflammation Federation Proc 11 424
49 WACHSTEIN M 1949 Reduction of potassium tellurite by living tissues Proc Soc Exptl Biol Med 72 175
50 WACHSTEIN M 1950 Histochemical demonstration of reducing activity in normal and leukemic blood and bone marrow cells Proc Soc Exptl Biol Med 73 306
51 WACHSTEIN M & E MEISEL 1954 Influence of experimental renal damage in histochemically demonstrable succinic dehydrogenase activity in the rat Am J Path 30 147
52 WACHSTEIN M 1946 Alkaline phosphatase activity in normal and abnormal blood and marrow cells J Lab Clin Med 31 1
53 PLUM C M 1950 Die alkalische Phosphatase in den Zellen des normalen and pathologischen Knochenmarkes und des peripheren Blutes Acta hematol 4 73
54 VALENTINE W N & W S BECK 1951 Biochemical studies on leukocytes I Phosphatase activity in health leukocytosis and myelocytic leukemia J Lab Clin Med 38 39
55 BECK W S & W N VALENTINE 1951 Biochemical studies on leukocytes II Phosphatase activity in acute leukemia chronic lymphatic leukemia and miscellaneous hematologic disorders J Lab Clin Med 38 245
55a STORTI L S PERUGINI & V ROSSI 1953 Quadro citoto pochimico delle fosfatasi nelle cellulè del sangue e degli organi emopoietici dell uomo normale Medicina (Parma) 3 333
56 RUTENBURG A M & A M SELIGMAN 1954 New method for the histochemical demonstration of acid phosphatase Proc 5th Ann Meet Histochemical Soc J Histochem Cytochem 2 445
57 RABINOVITCH M & L C U JUNQUEIRA 1948 Cytochemical demonstration of acid phosphatase in bone marrow smears Science 107 322
58 RABINOVITCH M & D ANDRUCCI 1949 A histochemical study of acid and alkaline phosphatase distribution in normal human bone marrow smears Blood 4 580
59 MONTAGNA W & C R NOBACK 1948 Cytochemistry of mast cells Anat Record 100 535
60 MORI T 1953 Histochemical study on the phosphatase distribution in rabbit bone marrow cells Tôhoku J Exptl Med 58 119
61 REIS J L 1937 Ueber die Aktivitaet der 5 Nucleotidase in den tierischen und menschlichen Geweben Enzymologia 2 183
62 WACHSTEIN M & E MEISEL 1952 Histochemical demonstration of 5-Nucleotidase activity in cell nuclei Science 115 652
63 SWENSEID M E P E WRIGHT & F H BETHELL 1952 Variations in nucleotidase activity of leukocytes Studies with leukemia patients J Lab Clin Med 40 515
64 CHIQUOINE A D 1953 *The distribution of glucose 6-phosphate in the liver and kidney of the mouse* J Histochem Cytochem 1 949
65 WACHSTEIN M & L MEISEL 1954 Unpublished observation
66 VOGEL F S 1951 A lipolytic enzyme in reactive histiocytes of guinea pigs with experimental encephalomyelitis J Exptl Med 93 305
67 GOMORI G 1953 Chloroacyl esters as histochemical substrates J Histochem Cytochem 1 469
68 ZAJICEK J B SYLVÉN & N DATTA 1954 Attempts to demonstrate Acetylcholine esterase activity in blood and bone marrow cells by a modified technique J Histochem Cytochem 2 115
69 FRIEDENWALD J S & B BECKER 1948 The histochemical localization of glucuronidase J Cellular Comp Physiol 31 303
70 SELIGMAN A M F C TSOU S H RUTENBURG & R B COHEN 1954 Histochemical

demonstration of D Glucuronidase with a synthetic substrate J Histochem Cytochem 2 209
41 FOLETTE J H W N VALENTINE & J S LAWRENCE 1952 The beta glucuronidase content of human leucocytes in health and in disease J Lab Clin Med 40 875

Discussion of the Paper

DOCTOR J PADAWER (*New York University New York N Y*) We have employed Sudan black B in the study of eosinophils in rat peritoneal fluid The speed of staining in our hands has been largely a function of the age of the saturated alcoholic solution of the dye With a freshly prepared solution used within a few days eosinophils are well stained within five minutes while after a few months the solution requires about an hour A nonstained core within the eosinophil granules is seen in large areas of some smears but not in other regions of the same preparation suggesting the possibility of a technique artifact *It should be emphasized that it is not sufficient to specify that* a smear has been stained with Sudan black B ' Our own experience has shown that on air-dried smears stained for one hour with a saturated solution of Sudan black B in 70 per cent ethanol only the eosinophil granules are visualized On the other hand if a propylene glycol solution (Chifelle and Putt) is used none of the eosinophils stains even after three days but mast cell granules now do even though they are not brought out by the alcoholic stain When Baker s formol-calcium fixative is used on air-dried smears prior to staining the mast cells stain even in the ethanolic Sudan black B but eosinophil granules are now negative It appears, therefore that mast cells contain a fatlike substance soluble in 70 per cent alcohol but not in propylene glycol and that the solubility in the former medium is lost after treatment with calcium ion Eosinophils on the other hand contain a fatty substance soluble in propylene glycol but not in 70 per cent alcohol This substance is also soluble in some aqueous fixatives even in the presence of calcium It would appear that conditions of staining must be reported in complete detail if they are to be of value to other investigators

44 VALENTINE W N W S BECK J H FOLETTE H MILLS & J S LAWRENCE 1952 Biochemical studies in chronic myelocytic leukemia polycythemia vera and other idiopathic myeloproliferative disorders Blood 7 959
45 SEABRA P 1955 Oxidase and lipase of the leukocytes Ann N Y Acad Sci 59(5) 1077
46 HOFFMANN G T A ROTTINO & K G STERN 1951 Demonstration of the Nadi re action of cytochrome oxidase activity in cells of the lymphoid and myeloid series obtained from normal individuals and patients suffering from Hodgkin s and other diseases Blood 6 1051
47 GREENSTEIN J P J WERNE A B ESCHENBRENNER & F M LEUTHARDT 1944 Chemical studies on human cancer I Cytochrome oxida e cytochrome c and copper in normal and neoplastic tissue J Natl Cancer Inst 5 55
48 REBUCK J W 1952 Enhanced nadireaction in lymphocytes in inflammation Fed eration Proc 11 424
49 WACHSTEIN M 1949 Reduction of potassium tellurite by living tissues Proc Soc Exptl Biol Med 72 175
50 WACHSTEIN M 1950 Histochemical demonstration of reducing activity in normal and leukemic blood and bone marrow cells Proc Soc Exptl Biol Med 73 306
51 WACHSTEIN M & E MEISEL 1954 Influence of experimental renal damage in histochemically demonstrable succinic dehydrogenase activity in the rat Am J Path 30 147
52 WACHSTEIN M 1946 Alkaline phosphatase activity in normal and abnormal blood and marrow cells J Lab Clin Med 31 1
53 PLUM C M 1950 Die alkalische Phosphatase in den Zellen des normalen and pathologischen Knochenmarkes und des peripheren Blutes Acta hematol 4 73
54 VALENTINE W N & W S BECK 1951 Biochemical studies on leukocytes I Phosphatase activity in health leukocytosis and myelocytic leukemia J Lab Clin Med 38 39
55 BECK W S & W N VALENTINE 1951 Biochemical studies on leukocytes II Phosphatase activity in acute leukemia chronic lymphatic leukemia and miscellaneous hematologic disorders J Lab Clin Med 38 245
55a STORTI E S PERUGINI & V ROSSI 1953 Quadro citoto pochimico delle fosfatasi nelle cellulè del sangue e degli organi emopoietici dell uomo normale Medicina (Parma) 3 333
56 RUTENBURG A M & A M SELIGMAN 1954 New method for the histochemical demonstration of acid phosphatase Proc 5th Ann Meet Histochemical Soc J Histochem Cytochem 2 445
57 RABINOVITCH M & L C U JUNQUERIRA 1948 Cytochemical demonstration of acid phosphatase in bone marrow smears Science 107 322
58 RABINOVITCH M & D ANDRUCCI 1949 A histochemical study of acid and alka line phosphatase distribution in normal human bone marrow smears Blood 4 580
59 MONTAGNA W & C R NOBACK 1948 Cytochemistry of mast cells Anat Record 100 535
60 MORI T 1953 Histochemical study on the phosphatase distribution in rabbit bone marrow cells Tôhoku J Exptl Med 58 119
61 REIS J L 1937 Ueber die Aktivitaet der 5 Nucleotidase in den tierischen und mensch lichen Geweben Enzymologia 2 183
62 WACHSTEIN M & E MEISEL 1952 Histochemical demonstration of 5 Nucleotidase activity in cell nuclei Science 115 652
63 SWENSEID M E P E WRIGHT & F H BETHELL 1952 Variations in nucleotidase activity of leukocytes Studies with leukemia patients J Lab Clin Med 40 515
64 CHIQUOINE A D 1953 The distribution of glucose 6-phosphate in the liver and kid ney of the mouse J Histochem Cytochem 1 949
65 WACHSTEIN M & E MEISEL 1954 Unpublished observation
66 VOGEL F S 1951 A lipolytic enzyme in reactive histiocytes of guinea pigs with ex perimental encephalomyeliti J Exptl Med 93 305
67 GOMORI G 1953 Chloroacyl esters as histochemical substrates J Histochem Cytochem 1 469
68 ZAJICEK J B SYLVÉN & N DATTA 1954 Attempts to demonstrate Acetylcholine esterase activity in blood and bone marrow cells by a modified technique J Histochem Cytochem 2 115
69 FRIEDENWALD J S & B BECKER 1948 The histochemical localization of glucuroni dase J Cellular Comp Physiol 31 303
70 SELIGMAN A M K C TSOU S H RUTENBURG & R B COHEN 1954 Histochemical

vantage of the tantalum grid method is that the medium can be changed without disturbing the cultures The pH of the medium is not critical and can vary between 6 5 and 9 0 without ill effect The health of the cultures is assessed quantitatively by counting the percentage of dead (pyknotic) lymphocytes present in film preparations or sections made at the end of the experiment

The culture medium which we have mostly used is adult rat serum with added glucose and this medium has given fairly satisfactory results the number of dead lymphocytes remaining below 2 per cent through four days Surprisingly it was found that the serum of 4-week-old rats whether autologous or homologous was much inferior to that of adult rats Rabbit serum and horse serum were not quite so good as adult rat serum but were better than the serum of young rats

By the systematic addition of various simple nutrients to Tyrode solution we have been able to develop some synthetic media which have given encouraging results So far the best results have been obtained with a relatively simple medium which contains only Tyrode salts glucose 19 amino acids cocarboxylase and p-aminobenzoic acid This medium has proved to be better *than any serum medium possibly because serum always contains adrenal* cortical hormones some of which are harmful to lymphocytes In the synthetic medium not only is lymphocyte survival somewhat better than in serum but a certain amount of cell differentiation occurs and we have rarely seen any ign of this phenomenon in serum This differentiation involves first the small lymphocytes many of which after four days *in vitro* have hypertrophied and turned into cells which must be called by some such name as hypertrophic lymphocytes monocytoid lymphocytes or polyblasts There seems little doubt that this represents a differentiation of small lymphocytes in the direction of monocytes or macrophages and that the phenomenon is essentially the same as that observed by Doctor Rebuck in his study of inflammation As is well known similar differentiation of small lymphocytes was reported many years ago by Maximow Bloom and others in tissue cultures of lymphoid tissue and in inflammation A second type of differentiation involves the reticulum cells many of which after four days *in vitro* have developed large nucleoli and some basophilia of the cytoplasm and appear to be turning into large lymphocytes I think these cells correspond to what Doctor Sundberg would call hemopoietic reticulum cells All intermediate stages between reticulum cells and large lymphocytes are found but we have not been able to convince ourselves that the total number of large lymphocytes increases Nevertheless we interpret these changes as the beginning of heteroplastic lymphopoiesis We have observed in the cultures that intermediate cells of this type may sometimes contain a few phagocytosed pyknotic lymphocytes in their cytoplasm which suggests to us that the ordinary phagocytic reticulum cells or histocytes differentiate into lymphocytes and we question the existence of a separate type of primitive nonphagocytic reticulum cell Incidentally Maximow held this view in 1924 but later he returned to a belief in primitive reticulum cells

These results the good survival of lymphocytes and the cell differentiations

EXPERIMENTS ON LYMPH NODES CULTURED *IN VITRO*

By O A Trowell

Medical Research Council Radiobiological Research Unit Harwell Berkshire England

In this investigation lymph nodes were cultured *in vitro* by a method of organ culture as distinct from tissue culture That is to say, the lymph node remains as an intact organ there is no outwandering of cells into the culture medium We have found that the reticulum cells will survive under a wide range of cultural conditions and for a long time The lymphocytes on the other hand, are extremely exacting in their cultural requirements and under the best conditions so far devised they will survive for eight days but not much longer

The lymph nodes used are the lumbar sacral and iliac of 4-week old rats The sacral and iliac are used whole but the lumbar nodes which are elongated structures are usually cut transversely into two or three pieces so that the cultures are fairly uniform in size each weighing about 2 mgm In these young rats, as Doctor Kindred has already reported lymphopoiesis occurs mainly in the thymus There is very little lymphopoietic activity in the lymph nodes which consist essentially of a network of reticulum cells packed full of small lymphocytes A few medium and large lymphocytes are present but no germinal centers and very little mitotic activity We have not obtained any growth of these lymph nodes *in vitro* and have aimed only at a satisfactory survival of the lymphocytes

The most important factor in insuring survival of the lymphocytes seems to be a good oxygen supply and to secure such a supply it is necessary first that the cultures are placed on the surface of the culture medium so that they are as close as possible to the gas phase and second that the gas phase is pure oxygen or oxygen with a small percentage of CO This is not to say that each lymphocyte requires a high oxygen tension for survival but it is only by maintaining a high oxygen tension at the surface of the culture that any oxygen at all can reach the cells in the center of the culture We have in fact shown by calculation that the oxygen tension at the center of the culture must be quite low So our findings are not necessarily in conflict with those of Doctor Osgood

The cultures may be placed on the surface of a semisolid culture medium such as plasma clot serum agar or cottonwool soaked in serum We have recently devised a better and simpler method in which the cultures are supported at the surface of a fluid medium on a grid made of fine tantalum wire gauze Tantalum is a nontoxic metal chemically almost as resistant as platinum and as strong as steel A piece of lens paper intervenes between the cultures and the wire grid The grid stands in a shallow dish of culture medium and after planting the cultures the dish is placed in a lucite chamber which is then filled with oxygen Normally a grid one inch square is used and this grid carries 20 cultures in 5 ml of medium The cultures consume a lot of glucose and produce lactic acid, so it is necessary to start with 0 4 per cent of glucose in the medium and to change the medium every two days An ad

vantage of the tantalum grid method is that the medium can be changed with out disturbing the cultures The pH of the medium is not critical and can vary between 6 5 and 9 0 without ill effect The health of the cultures is assessed quantitatively by counting the percentage of dead (pyknotic) lymphocytes present in film preparations or sections made at the end of the experiment

The culture medium which we have mostly used is adult rat serum with added glucose and this medium has given fairly satisfactory results the number of dead lymphocytes remaining below 2 per cent through four days Surprisingly it was found that the serum of 4 week-old rats whether autologous or homologous was much inferior to that of adult rats Rabbit serum and horse serum were not quite so good as adult rat serum but were better than the serum of young rats

By the systematic addition of various simple nutrients to Tyrode solution we have been able to develop some synthetic media which have given encouraging results So far the best results have been obtained with a relatively simple medium which contains only Tyrode salts glucose 19 amino acids cocarboxylase and p-aminobenzoic acid This medium has proved to be better than any serum medium possibly because serum always contains adrenal cortical hormones some of which are harmful to lymphocytes In the synthetic medium not only is lymphocyte survival somewhat better than in serum but a certain amount of cell differentiation occurs and we have rarely seen any sign of this phenomenon in serum This differentiation involves first the small lymphocytes many of which after four days *in vitro* have hypertrophied and turned into cells which must be called by some such name as hypertrophic lymphocytes monocytoid lymphocytes or polyblasts There seems little doubt that this represents a differentiation of small lymphocytes in the direction of monocytes or macrophages and that the phenomenon is essentially the same as that observed by Doctor Rebuck in his study of inflammation As is well known similar differentiation of small lymphocytes was reported many years ago by Maximow Bloom and others in tissue cultures of lymphoid tissue and in inflammation A second type of differentiation involves the reticulum cells many of which after four days *in vitro* have developed large nucleoli and some basophilia of the cytoplasm and appear to be turning into large lymphocytes I think these cells correspond to what Doctor Sundberg would call hemopoietic reticulum cells All intermediate stages between reticulum cells and large lymphocytes are found but we have not been able to convince ourselves that the total number of large lymphocytes increases Nevertheless we interpret these changes as the beginning of heteroplastic lymphopoiesis We have observed in the cultures that intermediate cells of this type may sometimes contain a few phagocytosed pyknotic lymphocytes in their cytoplasm which suggests to us that the ordinary phagocytic reticulum cells or histocytes differentiate into lymphocytes and we question the existence of a separate type of primitive nonphagocytic reticulum cell Incidentally Maximow held this view in 1924 but later he returned to a belief in primitive reticulum cells

These results the good survival of lymphocytes and the cell differentiations

described occur, only if both cocarboxylose and p aminobenzoic acid are present in the medium at about 10^{-5} M concentration Thiamine can be used in place of cocarboxylase but we think the results are not quite so good The p aminobenzoic acid can be replaced by p hydroxybenzoic acid or by folinic acid (citrovorum factor) but not by folic acid It is rather surprising that folinic acid was in no way superior to p aminobenzoic acid The following substances were investigated over a range of concentration and found to have no beneficial effect nicotinic acid, nicotinamide, pyridoxine, pyridoxal, riboflavin pantothenic acid, coenzyme A, meso inositol, choline, vitamin B_{12}, phosphocreatine glutamine, acetate formate In the absence of p-aminobenzoic acid addition of the purine and pyrimidine bases was definitely beneficial but in the presence of p aminobenzoic acid no further improvement was obtained This result would support the concept that the fundamental effect of the p-aminobenzoic acid is to promote purine and pyrimidine synthesis as it does in bacteria Addition of various purified plasma proteins and commercial preparations of proteoses and peptones were without benefit Occasional mitoses of reticular cells large lymphocytes or medium lymphocytes were seen even after six days in the synthetic medium but we have been unable to find any mitotic stimulant which would give us a genuine lymphopoiesis *in vitro*

We have been particularly interested in the sensitivity of small lymphocytes to ionizing radiation which is one of the fundamental problems of radiobiology and I should like to report briefly on our radiation studies When the whole animal is irradiated it appears that the small lymphocytes are the most radiosensitive cells in the body, but this finding does not prove that these cells are themselves peculiarly radiosensitive for some indirect effect of irradiation might be involved for example via the adrenal cortex It was important therefore to see if lymphocytes *in vitro* were very radiosensitive particularly as some earlier workers had claimed they were not First of all whole animals (4-week rats) were irradiated with X rays and the LD50 for the lymphocytes in the lumbar and sacral lymph nodes was determined and found to be 150 r The LD50 is here defined as the dose required to kill 50 per cent of the lymphocytes in five hours Lymph node cultures were now irradiated (in serum medium) and the LD50 was found to be 275 r So it appears that the lymph node lymphocytes are somewhat less sensitive *in vitro* than *in vivo* but even *in vitro* they are still probably the most radiosensitive cells in the body Actually this *in vivo in vitro* comparison cannot be made with any accuracy because the radiosensitivity depends on the prevailing oxygen tension in the cells, and this tension is probably higher *in vitro* than *in vivo* though the precise figure is not known in either case The fact that radiosensitivity depends on the oxygen tension prevailing in or around the cells at the time of irradiation was strikingly shown in our studies When the oxygen tension was reduced to zero over the few minutes of irradiation the LD50 rose to 3 400 r in other words the radiosensitivity was decreased by a factor of 12 Changing the CO concentration over a wide range did not influence sensitivity Reducing the pH below 6 5

reduced sensitivity as also did the addition of 0 02 M lactate We also irradiated whole blood *in vitro* and found the LD50 for the blood lymphocytes to be 1600 r so that blood lymphocytes are about six times less sensitive than lymph node lymphocytes *in vitro* We were also able to confirm an observation of Doctor Bloom that the scattered lymphocytes present in the intestinal villi are rather resistant to radiation their LD50 was 2 250 r, so they are about 15 times less sensitive than the lymph node lymphocytes *in vitro* Taken as a whole, these studies show that the radiosensitivity of lymphocytes is a variable which can be considerably influenced by environmental conditions and if the same is true of other mammalian cells this is a fact of some importance both for radiotherapy and atomic warfare

Finally we have investigated the action of cortisone on these lymph node cultures Synthetic compound E in the form of the free alcohol was added to the culture medium and it was found that lymphocytes were killed by quite low concentrations but the effect was rather slow in onset Some lymphocytes were killed in five hours but the peak death was not reached until 24 hours When cortisone is injected into a whole animal the peak death of lymphocytes in the lymph nodes occurs at six to eight hours and the slower effect in the cultures is difficult to explain We do not think that the rate of diffusion of cortisone into the culture is the limiting factor In this connection I was interested in Doctor Martin s observation that when he added cortisone to his neutrophil suspensions the inhibition of glycolysis began to appear only after four to six hours We found that a cortisone concentration of 3×10^{-7} M (0 1 μg/ml) definitely killed some lymphocytes and that 3×10^{-6} M concentration killed about 50 per cent of the lymphocytes in 24 hours Deoxycorticosterone had a slight effect Testosterone estradiol and progesterone had no effect Addition of purines and pyrimidines to the culture medium slightly protected the lymphocytes from the action of cortisone It is possible therefore that cortisone acts by interfering with purine or pyrimidine synthesis and there is a certain amount of evidence that radiation may act in the same way

The small lymphocytes of lymphoid tissue are peculiarly sensitive to the destructive action of ionizing radiation cortisone nitrogen mustard and a great variety of mitotic poisons The reason for this sensitivity remains to be discovered and we can suggest only that it is probably related in some way to the relative paucity in cytoplasm